Estimation of illicit drug use in Reykjavik by wastewater-based epidemiology

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Thesis for the degree of Philosophiae Doctor

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Fíkniefni í frárennsli frá Reykjavík

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Ágrip

Mælingar á fíkniefnum og lyfseðilsskyldum lyfjum í frárennslisvatni hafa undanfarin ár verið notaðar til þess að meta notkun efnanna. Aðferðafræðin byggir á þeirri kenningu að hægt sé að líta á frárennslisvatn sem samansafn þvagsýna frá heilu samfélagi. Áreiðanlegar niðurstöður eru fengnar á mjög fljótvirkan hátt án þess að inngripum sé beitt.

Aðal markmið þessarar rannsóknar var að setja upp og gilda greiningaraðferð fyrir fíkniefni og lyfseðilsskyld lyf í frárennsli frá Reykjavík. Taka þurfti mið af íslenskum aðstæðum eins og mikilli vatnsnotkun. Vandamál sem tengjast tapi á efnasamböndum, sérstaklega kannabínóíðum, eru þekkt. Samanburðartilraunir voru skipulagðar til þess að bera kennsl á þessa þætti og þróa verklýsingu í þeim tilgangi að lágmarka tap þessara efna. Niðurstöður sýndu að hægt er að koma að mestu í veg fyrir tap á efnum með því að forðast síun og sýringu á sýnum. Fastfasa súluskiljun (SPE) var notuð við úrhlutun fíkniefna og lyfja í frárennslisvatni frá Reykjavík með ásættanlegum heimtum. Háþrýstivökvagreinir tengdur tvöföldum massaskynjara (UPLC-MS/MS) var notaður við greiningu efnanna. Greiningaraðferðir voru gildaðar samkvæmt samþykktum viðmiðunarreglum sem sýndi fullnægjandi niðurstöður. Áreiðanleiki greiningaraðferða var staðfestur árlega með þátttöku í samanburðarrannsóknum milli evrópskra rannsóknastofa. Frárennslissýnum var safnað í Reykjavík á ellefu tímapunktum frá febrúar 2017 til júní 2020. Sýnum var safnað með sjálfvirkum sýnatökubúnaði í 24 klst. yfir sjö daga í hvert skipti. Algeng fíkniefni sem notuð eru á Íslandi voru valin til greiningar ásamt lyfseðilsskyldum lyfjum með þekkta misnotkun. Notkun efnanna var metin í mg/dag/1000 íbúa.

Langtímaþróun í fíkniefnanotkun í Reykjavik var metin með mælingum á frárennslisvatni. Notkun kókaíns hækkaði verulega frá 2017 til 2019 en hafði minnkað umtalsvert í júní 2020 á tímum kórónuveirunnar. Notkun bæði amfetamíns og metamfetamíns sýndu merki aukningar frá 2017 til 2020, en notkun amfetamíns var mun meiri en metamfetamíns. Notkun 3,4-methylene-dioxymethamphetamine (MDMA) var stöðug frá 2017 til 2020. Notkun kannabis var stöðug frá 2017 til 2019 en sýndi merki aukningar á tímum kórónuveirunnar árið 2020. Aukning í notkun kókaíns og MDMA sást einnig um helgar þegar borið var saman við aðra vikudaga og einnig á tónlistarhátíð sem haldin var í Reykjavík. Niðurstöður byggðar á mælingum á frárennslisvatni voru

bornar saman við aðra vísa að fíkniefnanotkun, fjölda mála vegna fíkniefnaaksturs og gögn um haldlagt magn fíkniefna. Þróun í notkun lyfseðilsskyldra lyfja í Reykjavik var einnig metin og borin saman við gögn úr Lyfjagagnagrunni. Niðurstöður sýndu stöðuga notkun kódeins, morfíns og tramadóls frá 2017 til 2019. Samanburður við Lyfjagagnagrunn sýndi sambærilegar niðurstöður þar sem notkun kódeins var mun hærri en notkun morfíns. Gögn Lyfjagagnagrunns og niðurstöður byggðar á mælingum á frárennsli sýndu í báðum tilfellum litla aukningu í notkun metýlfenídats.

Afkastamikil sýnameðhöndlunaraðferð var þróuð þar sem minna rúmmál frárennslissýna var úrhlutað samanborið við aðrar hefðbundnari aðferðir. Þessi aðferð var þróuð bæði fyrir fíkniefni og lyfseðilsskyld lyf. Mikill tímasparnaður felst í því að úrhluta minna rúmmál af sýni, samanborið við aðrar eldri aðferðir og fást þá niðurstöður á mun skemmri tíma. Frárennslisýni frá fjórum höfuðborgum á Norðurlöndum (Osló, Stokkhólmur, Reykjavík og Þórshöfn) voru úrhlutuð með þessari aðferð. Gögn frá Helsinki voru einnig fengin og þróun í notkun örvandi fíkniefna var metin milli borganna. Niðurstöður sýndu að amfetamínmagn í frárennsli var mest í Reykjavík og Stokkhólmi. Magn metamfetamíns í frárennsli var hæst í Helsinki og magn MDMA var mest í Osló. Magn kókaíns í frárennsli var sambærilegt í Osló, Stokkhólmi og Reykjavík en mun lægra í Helsinki. Augljós aukning í magni MDMA og kókaíns sást um helgar sem bendir til að notkun efnanna til afþreyingar sé vinsæl á Norðurlöndum.

Þróun í notkun fíkniefna og lyfja frá árinu 2017 til 2020 var metin með mælingum á frárennsli með góðum árangri ásamt samanburði við önnur lönd. Niðurstöður byggðar á mælingum á frárennslisvatni voru almennt sambærilegar gögnum yfir fíkniefnaakstur og haldlagningar. Hægt er að ná fram heildstæðari mynd af notkun fíkniefna í Reykjavík með því að sameina niðurstöður sem byggðar eru á mælingum á frárennsli og önnur gögn. Þetta getur veitt hagsmunaaðilum eins og lögregluyfirvöldum og heilbrigðisstarfsmönnum mikilvægar upplýsingar sem nota má í baráttunni gegn fíkniefnavandanum.

Lykilorð:

Fíkniefni, lyfseðilsskyld lyf, misnotkun, frárennsli, efnagreining.

Abstract

Wastewater-based epidemiology (WBE) has in recent years emerged as a reliable way to estimate the use of illicit drugs and pharmaceuticals at a community level. This methodology is based on the hypothesis that wastewater can be considered a pooled urine sample of a total population. This is a non-intrusive tool which provides objective results in near real-time.

The primary aim of this project was to adapt and validate a reliable analytical method for illicit drugs and pharmaceuticals in wastewater from Reykjavik. Icelandic conditions were taken into account, such as high residential water usage, to reach detectable levels. Known issues due to the loss of compounds, predominantly cannabinoids, had been associated with the pre-treatment of samples. Inter-laboratory experiments were set up to identify these issues and further develop a best-practice protocol. The results showed that loss of these compounds could be mostly prevented by avoiding filtration and acidification of samples. A solid phase extraction (SPE) method was adapted for the analysis of illicit drugs and pharmaceuticals in wastewater samples from Reykjavik with satisfactory recoveries. Ultra-performance liquid chromatography coupled to a tandem mass spectrometry (UPLC-MS/MS) was used for the analysis of samples. The analytical methods were validated according to published guidelines showing satisfactory results. During the course of this project, the reliability of the analytical methods was confirmed yearly with participation in a European-wide inter-laboratory study. Wastewater samples were collected in Reykjavik at eleven timepoints from February 2017 to June 2020. Using an automatic sampling device, 24h composite samples were collected over seven days at a time. Commonly used illicit drugs in Iceland were selected for analysis; amphetamine, methamphetamine 3,4-methylenedioxymethamphetamine (MDMA), cocaine and cannabis along with pharmaceuticals with known abuse potential were also chosen. The estimated use of the analytes of interest was expressed as mg/day/1000 inhabitants.

Temporal trends in illicit drug use in Reykjavik were successfully assessed using WBE. The results showed that cocaine use had increased extensively from 2017 to 2019 but had decreased considerably in June 2020 during the COVID-19 pandemic. Amphetamine and methamphetamine both showed signs of increased use between 2017 and 2020, but amphetamine use remained significantly higher. MDMA use was estimated to be stable from

2017 to 2020. Estimated cannabis use by WBE was stable from 2017 to 2019 but showed signs of an increase in 2020 during the pandemic. Trends between weekdays and during special events were also observed showing a significant increase in cocaine and MDMA use during weekends and a music festival held in Reykjavik. Further, a comparison was made with other indicators of drug use based on seizure data and driving under the influence cases. Trends in the use of pharmaceuticals in Reykjavik were also estimated and compared with data from the Prescriptions Medicines Register of Iceland, showing stable use of codeine, morphine, and tramadol from 2017 to 2019. Data by WBE showed comparable results with prescription data for codeine and morphine where codeine use was considerably higher. Both data from the Prescriptions Medicines Register and results based on wastewater analysis showed a small increase in methylphenidate use.

A high-throughput SPE micro-extraction method combined with large volume injection and post-loop mixing was developed for both illicit drugs and pharmaceuticals. By extracting smaller volumes of samples compared to other conventional methods, the time per sample was lowered considerably allowing for faster reporting of results. Samples from four Nordic capitals (Oslo, Stockholm, Reykjavik, and Torshavn) were analysed using SPE micro-extraction where spatial trends were successfully estimated alongside data obtained from Helsinki. The results showed high amphetamine loads in Reykjavik and Stockholm. Methamphetamine loads were highest in Helsinki and MDMA loads were highest from Oslo. Cocaine loads were similar from Oslo, Stockholm, and Reykjavik, but notably lower from Helsinki. A clear increase was observed in MDMA and cocaine loads during weekends indicating recreational use of the drugs.

Both temporal and spatial trends in illicit drug use were successfully estimated from 2017 to 2020 by using WBE. Overall, results by WBE were comparable with the other indicators of drug use. By complimenting data by WBE and other methods used to assess the use of drugs, a more comprehensive picture of the community drug abuse can be achieved. This could provide stakeholders such as the law enforcement or health care authorities valuable and useful information in their fight against drug abuse.

Keywords:

Illicit drugs, pharmaceuticals, drug abuse, wastewater, analysis.

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I want to dedicate this thesis to my true inspiration, my son Þráinn Löve.

Contents

List of abbreviations

ADHD	Attention deficit hyperactivity disorder
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
CV	Coefficient of variation
DDD	Defined daily doses
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray ionization
IUPAC	International Union of Pure and Applied Chemistry
LOQ	Limit of quantification
MDMA	3,4-Methylenedioxymethamphetamine
MRM	Multiple reaction monitoring
NIVA	Norwegian Institute for Water Research
PP	Polypropylene
RSD	Relative Standard Deviation
SCORE	Sewage analysis CORe group Europe
SPE	Solid phase extraction
SWGTOX	Scientific Working Group for Forensic Toxicology
THC	Delta-9-tetrahydrocannabinol
THCA	11-Nor-9-carboxy-delta-9-tetrahydrocannabinol
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
WBE	Wastewater-based epidemiology
WTP	Wastewater treatment plant

List of figures

List of tables

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV [as needed]):

I. Causanilles, A., Baz-Lomba, J.A., Burgard, D.A., Emke, E., González-Mariño, I., Krizman-Matasic, I., Li, A., **Löve, A.S.C.**, McCall, A.K., Montes, R., van Nuijs, A.L.N., Ort, C., Quintana, J.B., Senta, I., Terzic, S., Hernandez, F., de Voogt, P., Bijlsma, L., 2017. Improving wastewater-based epidemiology to estimate cannabis use: Focus on the initial aspects of the analytical procedure. Anal. Chim. Acta, 2(988), 27-33. doi: 10.1016/j.aca.2017.08.011

II. Baz-Lomba, J.A., **Löve, A.S.C.**, Reid, M.J., Ólafsdóttir, K., Thomas, K.V., 2018. A high-throughput solid-phase microextraction and post-loop mixing large volume injection method for water samples. J. Chromatogr. A, 1531, 32-38. doi: 10.1016/j.chroma.2017.11.051

III. **Löve, A.S.C.**, Ásgrímsson, V., Ólafsdóttir, K. Illicit drug use in Reykjavik by wastewater-based epidemiology. Submitted for publication in Sci. Total Environ in April 2021.

IV. **Löve, A.S.C.**, Baz-Lomba, J.A., Reid, M.J., Kankaanpää, A., Gunnar, T., Dam, M., et al., 2018. Analysis of stimulant drugs in the wastewater of five Nordic cities. Sci. Total Environ., 627, 1039-1047. doi: 10.1016/j.scitotenv.2018.01.274

Declaration of contribution

Paper I: Ana Causanilles and Lubertus Bijlsma designed the study. Arndís Sue Ching Löve contributed with results from a stability experiment on the major metabolite of cannabis as well as a sorption experiment to two different types of container surfaces. Arndís provided information on the methodology used for analysis as well as results on accuracy and precision of the method. Arndís participated in an inter-laboratory experiment along with seven other laboratories used to confirm the recommended order of preparatory steps for the analysis of cannabis by WBE. Arndís also took part in reviewing the manuscript before publication.

Paper II: The experimental methods were developed by Jose Antonio Baz-Lomba and Malcolm J. Reid. Arndís Sue Ching Löve contributed by conducting initial testing of the sample extraction method which included experiments on different wastewater matrixes. Arndís also performed sample extraction on wastewater samples from Oslo for the quantification of illicit drug and pharmaceuticals presented in the paper. Arndís took part in writing the methods chapter and final reviewing of the manuscript before publication.

Paper III: Arndís Sue Ching Löve adapted and validated the sample extraction method used for the analysis of wastewater samples from Reykjavik. Arndís performed all sample collections included in this paper. Arndís carried out the analysis and data processing. Valþór Ásgrímsson provided expert knowledge on seizure data and comparison with other indicators of illicit drug use. Kristín Ólafsdóttir provided her scientific expertise in analytical chemistry as well as knowledge on illicit drug use. Arndís, Kristín, and Valþór collaborated on the interpretation of results and Arndís wrote the manuscript.

Paper IV: Arndís Sue Ching Löve contributed to this paper by performing the sample extraction of wastewater samples from four Nordic capitals. The analytical methods were setup by Jose Antonio Baz-Lomba and Malcolm J. Reid (Paper II). Sampling performed at the four locations was organized by Arndís, Jose Antonio Baz-Lomba, and Maria Dam. Arndís and Jose Antonio Baz-Lomba performed the sample analysis. Results on the analysis of wastewater samples from Helsinki were provided by Aino Kankaanpää and Teemu Gunnar. The interpretation of results was made by Arndís as well as writing of the manuscript.

1 Introduction

The misuse of drugs poses a continuous threat to modern society due to their extensive negative effects on public health. The abuse of drugs can cause a wide range of health related harms and is directly linked to a variety of social consequences (United Nations Office on Drugs and Crime, 2020). Physiological effects are the primary causal factor of physical harm due to drug abuse. These effects can be characterized as either acute (e.g. respiratory depression due to opioids or fatal overdoses) or chronic (e.g. psychosis due to stimulants). The route of administration can also be a significant factor in the physical harm that drugs of abuse can cause, for example the risks associated with intravenous use compared to oral use. These risks are e.g. acute toxicity and the spread of blood borne viruses such as HIV and hepatitis (Nutt et al., 2007). A major adverse effect caused by drug abuse is physical dependence. Repeated use can increase physical tolerance and addiction and can subsequently cause withdrawal symptoms after the use has ceased (Degenhardt and Hall, 2012; Nutt et al., 2007). Negative social effects due to drug abuse are well known. These damaging consequences occur mainly through increased crime rates and high health-care costs. Intoxication in the form of neglect and violent behaviour also affects the family and social life of the abuser (United Nations Office on Drugs and Crime, 2020). The non-medical use of several substances has been banned by international drug control treaties due to these risk factors where the objective is to fight against their abuse with coordinated international measures (Nutt et al., 2007).

1.1 Commonly abused drugs

The most recent studies on the drug situation in Europe show that cannabis has the highest lifetime prevalence followed by cocaine, MDMA and amphetamines (European Monitoring Centre for Drugs and Drug Addiction, 2020). In Iceland, cannabis is the most commonly used illicit drug followed by amphetamine, cocaine, MDMA and methamphetamine (The Directorate of Health, 2012b; Tyrfingsson, 2019). The non-medical use of prescription drugs is also an increasing problem in Iceland (Tyrfingsson, 2019).

1.1.1 Cannabis

Cannabis is produced from the plant *Cannabis sativa* L. and is believed to be originated in central Asia (Baselt, 2017; European Monitoring Centre for Drugs and Drug Addiction, 2012; United Nations Office on Drugs and Crime, 2012). This plant is considered to be monotypic, with several subspecies based on chemical, morphological and typological characteristics. These characteristics are however not always apparent due to different environmental conditions (European Monitoring Centre for Drugs and Drug Addiction, 2012; United Nations Office on Drugs and Crime, 2009).

Cannabis contains the active ingredient delta-9-tetrahydrocannabinol (THC) (Baselt, 2017). The chemical structure of THC is shown in Figure 1. THC binds to cannabinoid receptors on the surface of neurons, which produce psychoactive effects (United Nations Office on Drugs and Crime, 2012). THC is highly lipophilic, and its distribution after cannabis administration is widespread in the body. THC is excreted in both faeces and urine in only trace amounts. THC is metabolized into two active monohydroxy compounds which do not reach significant concentrations in plasma. The active metabolite 11-OH-THC is rapidly oxidized into the major secondary metabolite 11-COOH-THC, also called 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THCA) (Baselt, 2017; Gracia-Lor et al., 2016).

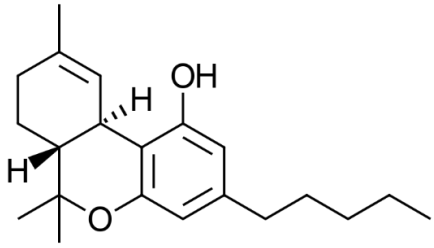

Figure 1. Chemical structure of THC.

The amount of THC varies throughout the plant with the highest concentration in the floral part, followed by the leaves and stem (European Monitoring Centre for Drugs and Drug Addiction, 2012). THC is produced in the glandular trichomes on the surface of the plant. These structures are distributed differently throughout the plant, which explains the variations in THC concentration. Their function is to protect the plant against herbivores by producing a glutinous and sticky mixture which immobilizes insects and prevents them from feeding on the plant (European Monitoring Centre for Drugs and Drug Addiction, 2012, 2019a).

Known adverse effects due to cannabis use include euphoria, relaxation, sensory alterations, time distortion, anxiety, and panic reactions (Hall and Solowij, 1998). Cannabis is mainly used in herbal form or as resin (European Monitoring Centre for Drugs and Drug Addiction, 2019a). The herbal form, also known as “marijuana”, or “weed”, consists of dried leaves or flower tops (United Nations Office on Drugs and Crime, 2012). Female cannabis plants contain the highest amounts of glandular trichomes and are therefore preferably harvested for herbal cannabis (European Monitoring Centre for Drugs and Drug Addiction, 2012; United Nations Office on Drugs and Crime, 2009). Cannabis resin, also known as “hashish”, is produced by sieving crushed cannabis, which is pressed into slabs, balls, or other shapes. This material can contain high concentrations of THC compared to herbal cannabis (European Monitoring Centre for Drugs and Drug Addiction, 2019a; United Nations Office on Drugs and Crime, 2012). Cannabis is commonly smoked in rolled cigarettes mixed with tobacco. Other forms of cannabis such as oils and edibles (foods or liquids) have also been introduced to the European market in recent years in increasing amounts (European Monitoring Centre for Drugs and Drug Addiction, 2019a).

The recreational use of cannabis as known today can be dated back to the 1960s. In the 1990s and 2000s, the consumption rates began to increase extensively in most European countries (European Monitoring Centre for Drugs and Drug Addiction, 2012; United Nations Office on Drugs and Crime, 2012). Cannabis is currently the most commonly used illicit drug in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2020).

1.1.2 Amphetamine

The central nervous system stimulant amphetamine was first introduced onto the market in 1935 under the trade name Benzedrine® for the treatment of narcolepsy, obesity, depression, and hypotension. Amphetamine later also became a medication for attention deficit hyperactivity disorder (ADHD) (European Monitoring Centre for Drugs and Drug Addiction and Europol, 2011). Amphetamine presently still prescribed for indications such as ADHD and narcolepsy (Heal et al., 2013; Karlstad et al., 2016). During the Second World War, amphetamine was used to produce wakefulness with large quantities being manufactured. Consequently, the abuse and misuse of prescribed amphetamine became recognized in the 1950s. Illegal production of the drug has been known since the 1970s (European Monitoring Centre for

Drugs and Drug Addiction, 2010; European Monitoring Centre for Drugs and Drug Addiction and Europol, 2011; Heal et al., 2013).

Amphetamine belongs to the chemical class of phenethylamines. The molecule of amphetamine was first synthesized in 1887 and contains a single chiral centre with two active forms, the dextro- and levo-isomers (Baselt, 2017). The chemical structure of amphetamine is shown in Figure 2. This compound increases the synaptic concentrations of monoamine neurotransmitters such as dopamine and norepinephrine and therefore central stimulant activity (Baselt, 2017; Heal et al., 2013). Amphetamine is mainly excreted as the parent compound in urine accounting for approximately 30% of a dose (Baselt, 2017). Excretion rates of amphetamine are highly dependent on urine acidity and could increase to 74% in acidic urine or decrease to 1% in alkaline urine (Baselt, 2017; Postigo et al., 2008; van Nuijs et al., 2011a; van Nuijs et al., 2011b).

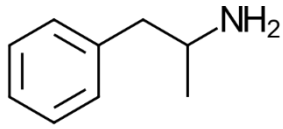

Figure 2. Chemical structure of amphetamine.

Amphetamine passes the blood-brain barrier and can cause a variety of dose-dependent behavioural changes. Known adverse effects of the drug include increased excitation, anorexia, hyperactivity, loss of sleep, nervousness, and cardiac and gastrointestinal effects. Pleasurable effects such as increased energy, wakefulness, elation and euphoria are also known which could encourage the patient to abuse the drug (Berman et al., 2009; European Monitoring Centre for Drugs and Drug Addiction, 2010). Drug abusers use several times higher amounts of the drug by snorting or intravenous injection compared to oral therapeutic use (European Monitoring Centre for Drugs and Drug Addiction, 2010). Due to the abuse potential of the drug, prescribers in many cases have turned to other alternatives such as methylphenidate for treatment of ADHD (Heal et al., 2013).

Since the 1990s the popularity of non-medical use of amphetamine has increased in Europe. Produced mainly in the Netherlands, Belgium and Poland, the availability of amphetamine is rising leading to increased detection in many countries. Amphetamine has been known to be more commonly used in Northern Europe compared with Southern parts (European Monitoring Centre for Drugs and Drug Addiction, 2020; González-Mariño et al., 2020).

1.1.3 Methamphetamine

The central nervous system stimulant methamphetamine was first synthesized in 1919. This substance has been clinically used for indications such as obesity, narcolepsy, and ADHD since the 1930s. The abuse of methamphetamine dates back to the Second World War and was prescribed extensively in the 1950s and 1960s (Anglin et al., 2000; Baselt, 2017). In the 1990s the recreational use of methamphetamine began to increase significantly worldwide (Galbraith, 2015).

Methamphetamine is a N-methyl derivative of amphetamine and therefore belongs to the chemical class of phenethylamines (Baselt, 2017). The chemical structure of methamphetamine is shown in Figure 3. Methamphetamine is mainly excreted unchanged through urine accounting for 43% of a dose. The compound also goes through some degree of N-demethylation to amphetamine which accounts for 4-7% of a dose. Like for amphetamine, excretion rates of unchanged methamphetamine are highly dependent on urine acidity (Baselt, 2017; Postigo et al., 2008; van Nuijs et al., 2011a; van Nuijs et al., 2011b).

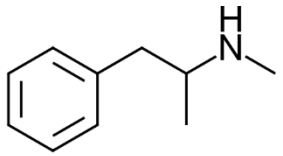

Figure 3. Chemical structure of methamphetamine.

Being a derivative of amphetamine, this drug has similar physiological effects on the central nervous system (Baselt, 2017). These effects include heightened alertness, euphoria, increased energy, hallucinations, depression, suicidal tendencies, anxiety and aggression (Anglin et al., 2000; Galbraith, 2015). This drug can be smoked, snorted, or injected. Methamphetamine is known to be highly addictive, especially after prolonged use at high levels. The addiction of the drug has been known to take place more rapidly compared to cocaine. The high potency of methamphetamine compared to amphetamine leads to persistent negative behavioural symptoms in patients with high tolerance (Galbraith, 2015).

Methamphetamine is mainly produced in Czechia and to some degree in the Netherlands (European Monitoring Centre for Drugs and Drug Addiction, 2020). The methamphetamine market in Europe is small compared to other stimulant drugs such as amphetamine (European Monitoring Centre for Drugs

and Drug Addiction and Europol, 2019). For the last decade, the methamphetamine market in Europe has been relatively stable and remained mainly in central Europe. Nevertheless, recent studies indicate that the availability of the drug is increasing and also spreading geographically through Europe (European Monitoring Centre for Drugs and Drug Addiction and Europol, 2019).

1.1.4 3,4-methylenedioxymethamphetamine

The illicit drug MDMA, also called “ecstasy”, was first synthesized in 1914 (Baselt, 2017; Rochester and Kirchner, 1999). In the 1950s, this drug was used to lower inhibitions in patients who were undergoing psychoanalysis and in the 1970s, to complement psychotherapy (Baselt, 2017; Rochester and Kirchner, 1999). In the 1980s, the recreational use of the drug became widespread both in the United States and Europe in association with the dance music scene (European Monitoring Centre for Drugs and Drug Addiction, 2016b).

MDMA is a ring substituted derivative of methamphetamine and belongs to the chemical class of substituted phenethylamines (European Monitoring Centre for Drugs and Drug Addiction, 2016b). The chemical structure of MDMA is shown in Figure 4. MDMA is excreted mainly as unchanged drug. The drug is both metabolized by N-demethylation to methylenedioxyamphetamine (MDA) and forms mono- and dihydroxy derivatives. MDMA accounts for 26% of an oral dose in urine with other metabolites in smaller proportions (Baselt, 2017).

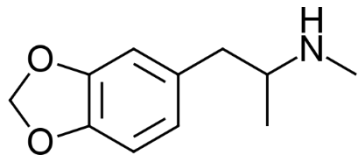

Figure 4. Chemical structure of MDMA.

MDMA can cause adverse effects such as confusion, anxiety, depression, hyperthermia, hypertension and cardiac arrhythmia (Baylen and Rosenberg, 2006). The substance is also known to produce physical effects such as increased sense of energy and sexual arousal and psychological effects such as euphoria, increased sociability, sexual arousal, and sensory perception. These effects are considered desirable to the user and encourage abuse of the drug (Baylen and Rosenberg, 2006; Kalant, 2001). MDMA is consumed

orally in tablets, which have various shapes and designs, or in crystal form (European Monitoring Centre for Drugs and Drug Addiction, 2020, 2016b).

The number of MDMA seizures in Europe has been on an upward trend since 2010, more commonly seized in Northern and Eastern Europe. However, in many European countries the prevalence of use has been either on the decline or shows stable estimates (European Monitoring Centre for Drugs and Drug Addiction, 2020).

1.1.5 Cocaine

Cocaine is found and extracted from the leaves of the plant *Erythroxylum coca* and *Erythroxylum novogranatense*, a tropical shrub originated in South America. Cocaine is almost entirely manufactured in Bolivia, Colombia and Peru where it plays an important cultural role where the substance has a spiritual, therapeutic and social purpose (Baselt, 2017; European Monitoring Centre for Drugs and Drug Addiction, 2016a). Cocaine was first isolated in 1855 and was used medically for anaesthesia (Baselt, 2017).

Cocaine belongs to the chemical class of tropane alkaloids which are naturally occurring compounds containing one or more nitrogen atoms and a tropane ring system (Kohnen-Johannsen and Kayser, 2019). The chemical structure of cocaine is shown in Figure 5. Only 1-9% of a cocaine dose is excreted unchanged through urine, depending on the urine acidity. Benzoylecgonine and ecgonine methyl ester are major metabolites of cocaine and represent 35-54% and 32-49% of the dose, respectively (Baselt, 2017). However, the excretion percentage of cocaine is highly dependent on the route of administration as the urinary excretion varies between oral, smoked, intravenous, and intranasal administration ranging from 6.5% (smoked administration) to 55% (oral administration) (Baselt, 2017; Castiglioni et al., 2013; Cone et al., 1998).

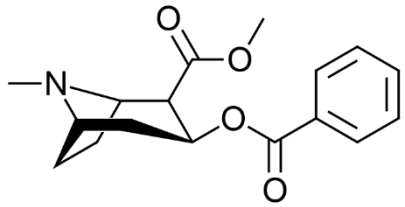

Figure 5. Chemical structure of cocaine.

Cocaine is abused for its stimulant effects on the central nervous system. Cocaine is known to cause adverse effects such as anxiety, agitation, irritability, confusion, hyperactivity, diaphoresis and cardiovascular effects (Baselt, 2017). This substance is abused in two forms. Firstly, cocaine in the form of a hydrochloric salt which is the most common form. The hydrochloric salt is mainly snorted through the nose or injected intravenously. Secondly, crack cocaine is a free base form of cocaine, which is smoked (European Monitoring Centre for Drugs and Drug Addiction, 2016a).

Cocaine is a commonly used stimulant in Europe, mainly in southern and western countries. In recent years, cocaine use has been on the rise in Europe showing record-high numbers of seizures. The purity of cocaine has not been higher in Europe in a decade causing the drug to have an increasing role in Europe´s drug problem (European Monitoring Centre for Drugs and Drug Addiction, 2020).

1.1.6 Pharmaceuticals

The misuses of opioids continue to be a serious threat to human health in Europe. As of 2020, 82% of fatal overdoses can be attributed to this class of compounds (European Monitoring Centre for Drugs and Drug Addiction, 2020). In the past two decades, the misuse of prescription drugs such as opioids and methylphenidate has increased in Iceland reaching record-breaking numbers in 2018 (Tyrfingsson, 2019).

The number of patients in Iceland addicted to opioids seeking treatment for their addiction was stable from 2005 to 2014 but increased by 34% from 2015 to 2018. The number of patients who inject opioids intravenously have increased also by 140% from 2010 to 2018 (Tyrfingsson, 2019). These drugs have previously been obtained mainly through prescriptions from doctors in Iceland, but in recent years a shift has been observed where illegally imported opioids have been entering the market (National Police Commissioner of Iceland, 2019; Tyrfingsson, 2019).

The number of new users of the drug methylphenidate, used to treat ADHD, increased by 78% from 2012 to 2017 (Jóhannsson et al., 2018). According to the Icelandic Prescription Medicines Register, defined daily doses (DDD) of filled prescriptions of methylphenidate have also increased in recent years. Over the past decade, the prevalence of methylphenidate used for the treatment of ADHD among adults has risen the most in Iceland compared to other Nordic counties (Geirs et al., 2014; Karlstad et al., 2016; Zoëga et al.,

2011). The prevalence of methylphenidate use in Iceland has also increased significantly among adults and most noticeably patients aged 19-24 (Geirs et al., 2014). A descriptive population-based study demonstrated that methylphenidate is the most commonly used intravenous drug in Iceland and users preferred the drug to other substances of abuse such as amphetamines and opioids (Bjarnadottir et al., 2015). Large amounts of methylphenidate have found their way into the illegal drug market in Iceland, followed by a rising numbers of people with pharmacological drug dependencies (Tyrfingsson, 2019).

1.2 Wastewater-based epidemiology

Detailed information on the use and availability of illicit drugs is crucial in order to shape effective actions against it (European Monitoring Centre for Drugs and Drug Addiction, 2020). Currently, such information is mainly obtained through population-based survey methods, consumer interviews, drug seizures and medical data (Reid et al., 2012). When estimating community drug use, several methodological challenges can arise such as reporting bias and under-reporting. Illicit drug use is stigmatized as well as illegal which can lead the consumer to give false information about their drug use. Drug users can also be unaware of the type of drug they have consumed (Castiglioni et al., 2014; Tyrfingsson, 2019). Furthermore, these survey methods can be time consuming where a considerable amount of time can pass between the collection of data to the reporting of results, which can lead to high costs (Hernández et al., 2018; van Nuijs et al., 2011b). Due to these limitations, it is important to develop alternative approaches with realistic and comparable estimations to compliment those previously used. Indicators from multiple sources is vital in order to gain a comprehensive picture of the illicit drug use of a community (European Monitoring Centre for Drugs and Drug Addiction, 2020). WBE has shown to be an effective way to estimate illicit drug use in a both detailed and rapid way.

1.2.1 Principles of wastewater-based epidemiology

The methodology of WBE is based on the hypothesis that wastewater could be considered a pooled urine sample of a large population and used as a non-intrusive tool to determine the drug use of a community (Daughton, 2001). An overview of the WBE approach is shown in Figure 6.

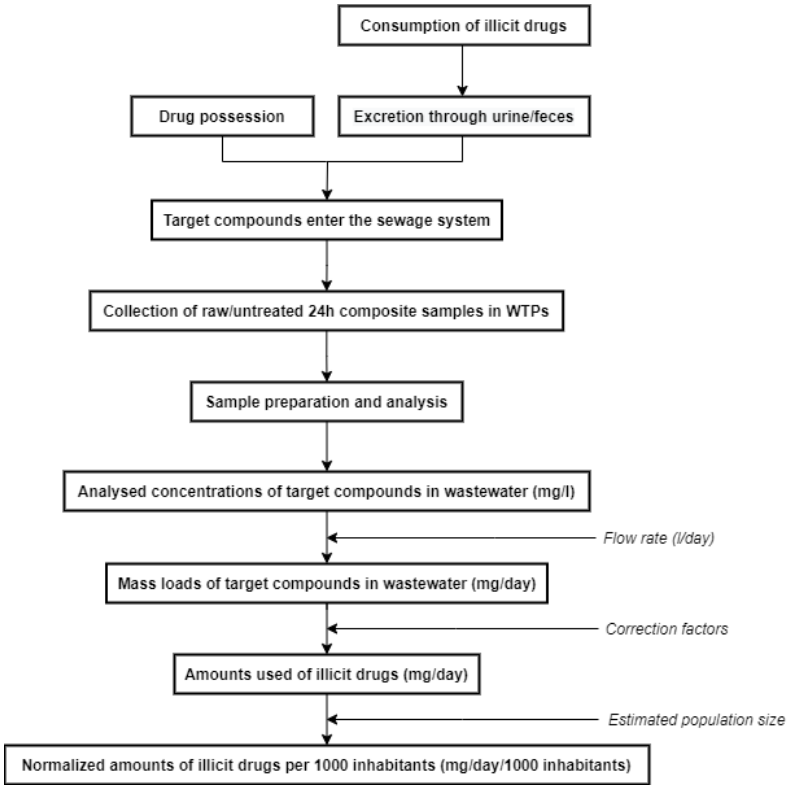

Figure 6. An overview of the WBE approach.

Illicit drugs and pharmaceuticals enter the body through various routes of administration, e.g. orally, intravenously or intranasally (Gracia-Lor et al., 2016). After consumption, the drugs are eliminated from the body through excretion. Illicit drugs and pharmaceuticals are either excreted as the parent compound or metabolite in urine or faeces. Excreted compounds ultimately enter the sewage system where samples are collected in wastewater treatment plants (WTP) (Zuccato et al., 2005a). Sample collection is conducted for 24 hours at a time, often for 7 days. Wastewater samples need to go through extensive sample preparation before analysis of chosen drug target residues, both to clean the sample of interfering compounds and to concentrate the analytes to detectable levels. Sensitive instruments are needed to detect the drug target residues. The analysed concentrations of drugs in wastewater can be back-calculated to used amounts in mg/day/1000 inhabitants (van Nuijs et al., 2011a). This methodology provides results in a short amount of time which

enables a near real-time comparison. WBE therefore provides an objective way to estimate both temporal and spatial trends in drug use.

1.2.2 History of wastewater-based epidemiology

Originally hypothesized by Daughton (2001), the methodology of WBE was first applied by Zuccato et al. (2005) where cocaine was analysed in surface waters from the river Po in Italy. This new approach gave promising and reproducible results in a short amount of time. Validated multi-residue analytical methods for illicit drugs in wastewater were subsequently introduced (Castiglioni et al., 2008; Castiglioni et al., 2006).

Zuccato et al. (2008) applied this approach to wastewater samples collected during a 7-day period from three locations in Europe. The results showed that by using WBE it is possible to monitor weekly fluctuations in illicit drug use, for example to detect trends in use during weekends. Further, the results showed that the methodology could be used to effectively compare illicit drug use between different locations in near real-time. Since then WBE has gained worldwide attention and has been applied in many countries around the world (Been et al., 2016; Bishop et al., 2020; Bramness et al., 2015; Bruno et al., 2018; Huerta-Fontela et al., 2008; Kankaanpää et al., 2016; Lai et al., 2016a; Li et al., 2019; Terzic et al., 2010; van Nuijs et al., 2011b; Yargeau et al., 2014).

Thomas et al. (2012) presented the simultaneous analysis of illicit drugs in wastewater from 19 European countries. For the first time, sample collection, analysis and data processing were coordinated by using a harmonized protocol. The illicit drug use of over 15 million individuals was determined over a period of one week, which proved to be in good accordance with official figures obtained from prevalence data. In 2014, both spatial and temporal trends in illicit drug use in 42 European cities were further explored (Ort et al., 2014). This study gave information on the illicit drug use of 24 million individuals that was generally in accordance with other indicators of drug use. Furthermore, the largest WBE study to date incorporated data from 120 cities obtained from 2011 to 2017 (González-Mariño et al., 2020). These multi-city studies successfully estimated illicit drug use on a large scale, showing both temporal and spatial differences, complimenting other methods of assessment (González-Mariño et al., 2020; Ort et al., 2014; Thomas et al., 2012).

Country-wide trends in illicit drug use have been estimated where wastewater samples have been collected in large parts of countries in

coordinated efforts (Kankaanpää et al., 2016; Lai et al., 2016a). With high population coverage, more representative results can be achieved as well as enabling the comparison of illicit drug use between rural areas and bigger cities (Kankaanpää et al., 2016). Longitudinal studies have also been conducted where the collection of wastewater samples has been carried out frequently (within days or weeks) at the same location. These studies give a more comprehensive picture of population drug use where emerging trends can be identified (Gunnar and Kankaanpää, 2019; Krizman-Matasic et al., 2019; Lai et al., 2016b).

WBE has been applied to smaller populations such as schools, prisons and workplaces (Gushgari et al., 2018; Lai et al., 2013; Panawennage et al., 2011; Postigo et al., 2011; Zuccato et al., 2017). This technique is both non-incriminating and not personally invasive compared to individual testing or population surveys (Panawennage et al., 2011). Illicit drug use in prisons by using WBE was investigated for the first time in 2011 with the intent to estimate the effectiveness of preventative measures taken to inhibit drug entry (Postigo et al., 2011). Illicit drug use of student populations has also been explored in both high schools and collages (Burgard et al., 2013; Gushgari et al., 2018; Panawennage et al., 2011; Zuccato et al., 2017). Although a promising tool to estimate drug abuse in schools in a non-intrusive way, various limitations were presented. These include the difficulty in distinguishing between the consumption by the students or faculty, the limitation of only detecting drugs which are consumed and excreted at the school, and difficulties retrieving representable samples (Zuccato et al., 2017). WBE studies have also focused on illicit drug use during special events such as music festivals and have been used to confirm recreational use (Lai et al., 2013). Results have been shown to be consistent over time and therefore could be used to study the impact of drug intervention programs (Lai et al., 2013; Mackuľak et al., 2019).

Drug use indicators such as data from prescription drug databases, information on driving under the influence cases, population surveys and seizure data have been used to compliment results by WBE (Been et al., 2015; Bruno et al., 2018; Kankaanpää et al., 2016; Reid et al., 2012). Data generated by WBE compared to other indicators of drug use provides a more detailed picture of the drug usage (Kankaanpää et al., 2016; Reid et al., 2012). The importance of utilizing information obtained through WBE in collaboration with stakeholders was emphasized and demonstrated by Gunnar et al. (2019). Data by WBE can for example be used in strategic decision making based on the size and structure of a drug market or as an early warning system on local hot spots and emerging new drugs (Gunnar and Kankaanpää, 2019).

The European-wide network Sewage analysis CORe group Europe (SCORE) was established in 2010. This collaboration between European laboratories in the field of WBE is to ensure the quality of data by improving and harmonizing analytical procedures with a common protocol of action. The SCORE group organized for the first time an inter-laboratory comparison study in 2011 as a quality control system for laboratories (van Nuijs et al., 2018). In 2019, these studies were performed in 29 countries and 86 cities showing over 80% of results being satisfactory (SCORE, 2020; van Nuijs et al., 2018).

1.2.3 Sample preparation and analysis

The analysis of drug target residues in wastewater samples has a vital role in the application of WBE. Sensitive analytical equipment is needed to detect the compounds of interest which are normally in the ng/L range. The preconcentration of wastewater samples is often necessary to reach these levels (van Nuijs et al., 2011a).

The most commonly reported sample extraction method for illicit drugs and pharmaceuticals in wastewater is SPE (van Nuijs et al., 2011a). This method is considered suitable for the preconcentration and purification of the analytes. SPE is most often based on disposable cartridges which are packed with a solid phase sorbent that retains the drug target residues. Wastewater samples are loaded onto these cartridges which causes the analytes of interest to adsorb to the stationary phase. The undesired contaminants are washed away before eluting analytes from the cartridges with suitable solvents or solvent mixtures. The eluted analytes are collected and analysed (Andrade-Eiroa et al., 2016). The appropriate sample extraction method is an extremely important step in removing interfering components from the sample matrix (van Nuijs et al., 2011a). The general procedure of SPE is shown in Figure 7.

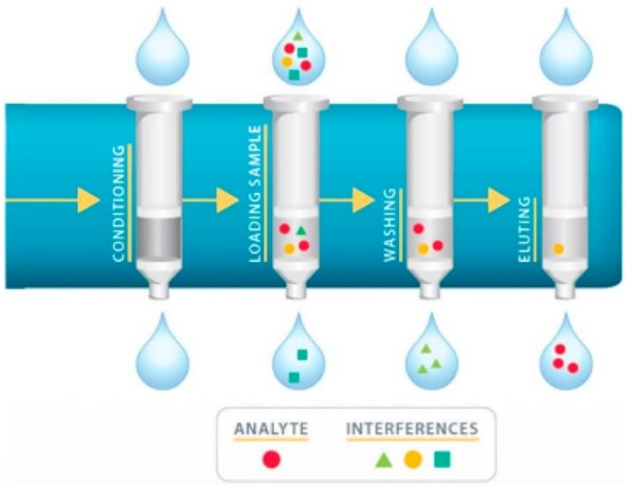

Figure 7. General steps for the SPE procedure (Andrade-Eiroa et al., 2016).

UPLC-MS/MS has been predominantly used for the analysis of drug target residues in wastewater (Hernández et al., 2018). This instrument is both highly sensitive and selective. The UPLC system is used to separate the analytes of interest, often with reversed phase columns and a mobile phase consisting of a mixture of water and an organic solvent (van Nuijs et al., 2011a). After separation, the analytes of interest travel through an ion source. Electrospray ionization (ESI) is a common ionization technique where charged molecules are produced. These molecules are subsequently transferred under high vacuum through the three quadrupoles of the mass spectrometer (Banerjee and Mazumdar, 2012; Fenn, 2003). The charged molecules selectively pass through the first quadrupole based on a specific mass to charge ratio. The selected mass to charge ions, called precursor ions, then pass through the second quadrupole called the collision cell. There, argon gas enters as a collision gas which causes the precursor ions to fragment into several product ions. In the third quadrupole, these product ions are scanned according to their mass to charge ratios. This transition from precursor ion to product ion is highly specific and acts as a fingerprint of the compound in question (Grebe and Singh, 2011; Vogeser, 2003). The design of a tandem mass spectrometer is shown in Figure 8.

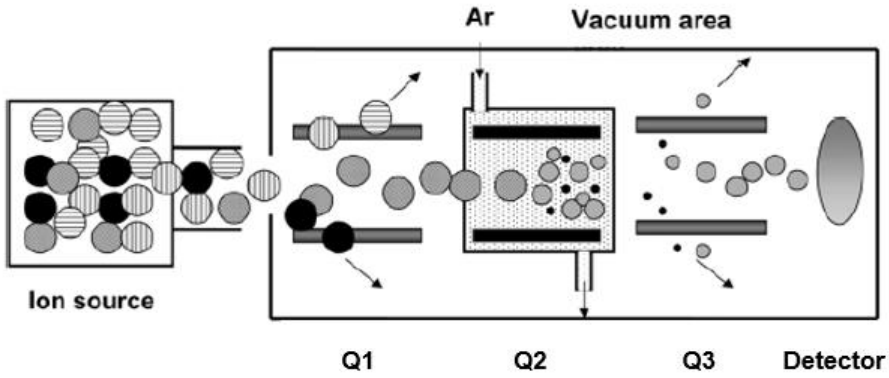

Figure 8. Design of a tandem mass spectrometer. Ar=argon gas, Q1=quadrupole 1, Q2= collision cell, Q3=quadrupole 3 (Vogeser, 2003).

1.2.4 Back-calculations

When concentrations of drug target residues in wastewater are back-calculated to used amounts, several parameters are incorporated. The flow rate of the wastewater stream is considered and is obtained with regular measurements in the WTPs throughout the day. Population size of the catchment area is incorporated where the estimated use is normalized to 1000 inhabitants. This enables the comparison of spatial trends. Various methods are used to estimate the population of the wastewater catchment (van Nuijs et al., 2011a; Zuccato et al., 2005b). The most common ways to assess population size are documented census numbers or estimations based on measurements of hydro-chemical parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), and ammoniacal nitrogen.

Correct selection of a drug target residue is important, depending on the excretion of the compound. A correction factor is incorporated into the back-calculations considering the different excretion rates of drug target residues. This correction factor includes the molecular mass ratio of the parent compound, and metabolite if applicable, and the excretion percentage of the drug target residue (Castiglioni et al., 2014; Gracia-Lor et al., 2016; Zuccato et al., 2008).

1.2.5 Possible uncertainties

Like other indicators of illicit drug use, WBE has potential limitations that are required to be addressed appropriately. These uncertainties can be related to e.g. the sample collection, stability of target residues in the wastewater and analytical methods or parameters used to back-calculate measured concentrations (Castiglioni et al., 2013; Lai et al., 2011; Zuccato et al., 2008).

The stream of the wastewater can vary substantially during the day. To avoid systematic under- or overestimations of results due to these fluctuations it is recommended to use volume- or flow-proportional sampling. When volume-proportional sampling is used, sampling intervals vary depending on the flow rate, but the volumes of individual samples are constant. Flow-proportional collects samples at constant intervals but the volumes of individual samples are variable depending on the flow rate. Both sampling techniques are based on input from flow meters. If only time-proportional (using constant volumes and time intervals) sampling equipment is used, the temporal resolution can be optimized to address flow rate variations. When planning the sample collection, it is also important to take into account any special event occurring during that time, such as music festivals or holidays, as they can affect patterns of use (Castiglioni et al., 2013). Other factors such as exfiltration from the sewage system and storage conditions during sample collection should also be taken into account to avoid underestimation of results (Castiglioni et al., 2013; McCall et al., 2016; Zuccato et al., 2008).

The in-sewer and in-sample biotransformation of drug residues in wastewater can increase the uncertainty of measurements. The transport of the wastewater through the sewer system can transform the chemical identity. These effects vary between sewers based on environmental conditions such as the sewer design, sorption to particulate matter, pH, temperature, flow rate, and dwell time in the wastewater. Currently there is limited information available on the effects of these factors on the transformation of residues and further research is needed (McCall et al., 2016). In-sample stability of residues can also be compromised if preventative measures are not taken. Several steps can be taken to minimize stability issues of analytes in collected wastewater samples. Appropriate storage (-20°C) should be ensured as soon as possible after sample collection if analysis is not performed immediately. It is also important to determine the influence of freeze/thaw cycles on the stability of compounds. The addition of labelled internal standards should be performed either before the samples are stored or immediately after they have been thawed. Other strategies such as acidification of samples or filtration can

also be considered depending on the analyte (Causanilles et al., 2017; McCall et al., 2016).

Known uncertainties have been associated with the analysis of biomarkers in wastewater. The correction of matrix effects is an important factor which need addressing to improve analytical performance. To prevent these effects, isotope dilution mass spectrometric method of quantification should be used with corresponding labelled internal standards for each analyte (Castiglioni et al., 2013). The participation in inter-laboratory studies can be used to further validate the reliability of measurements (van Nuijs et al., 2018).

Illicit drugs and pharmaceuticals are excreted either unchanged and/or as metabolites, mainly through urine or faeces. The excretion percentage of the analyte of interest is used in the back-calculations and has been linked to known uncertainties. Pharmacokinetic information on excretion rates of illicit drugs is scarce. The current available data is often based on a limited number of studies with few healthy participants. Further, excretion rates can vary between individuals, route of administration, frequency of use and other factors (Castiglioni et al., 2013). It is important to select the correct excretion rates and appropriate analytes to minimize uncertainties in back-calculations. The most preferred route of administration of an illicit drug can vary between countries. It is therefore critical to carefully consider the selection of correction factors to ensure the reliability of the estimations taking into account different routes of administration used by the target population (Castiglioni et al., 2013; Gracia-Lor et al., 2016).

A recognized challenge of WBE is how to best estimate the population size contributing to the wastewater sample (Castiglioni et al., 2013). Population statistics by residency can be insufficient as the exact number of people contributing to each site is likely to vary with time, e.g. due to tourism, commuting patterns, seasonal variability or special events (Lai et al., 2011). Several different approaches have been presented to accurately estimate the population size including measurements of organic compounds, prescribed pharmaceuticals and other biomarkers (Lai et al., 2015; Lai et al., 2011; van Nuijs et al., 2011b). Mobile data has also been used to estimate the dynamic population size where temporal variability can be taken into account (Baz Lomba et al., 2019; Thomas et al., 2017). The appropriate method to best estimate the population size of a catchment must be evaluated based on the resources available.

1.2.6 Ethical guidelines

The need for ethical guidelines when applying WBE has been expressed (Prichard et al., 2014). Known ethical issues have occurred in self-reported population studies where data on drug use is collected from individuals. To avoid these issues, the participants are required to provide their informed consent and the protection of sensitive information must be ensured. These ethical issues do not apply to WBE as the combined urine of a community of thousands cannot be used to identify the drug use of an individual. In the past, ethical boards have concluded that WBE studies involve low if any ethical issues, at least based on large populations. Although the ethical risks are low, there is a need for ethical guidelines that can address minor issues with relatively simple procedures (Prichard et al., 2014).

In 2015, ethical guidelines were published for the first time (Prichard et al., 2015). The aim of these guidelines was to define ethical risks for WBE research and to recommend strategies on how they can be minimized. These risks can arise from various sources such as incorrect or exploitative journalism, which can result in emotional harm for vulnerable groups and cause stigmatization. WBE research in smaller catchments such as prisons, schools, or workplaces, can cause potential ethical risks. Anti-drug strategies by prison authorities can cause negative effects to participants who did not provide their informed consent. The reputation of schools and workplaces can also be harmed, which could damage their economic status. In these instances, it is important to gain consent from relevant stakeholders, e.g. prison directors or school principals. Several other ways to minimize ethical risks are proposed in the guideline. These strategies include anonymizing data to prevent results being associated to e.g. certain schools, workplaces, or suburbs. Appropriate and effective representation of results to the media is vital to prevent misinterpretation of findings. WBE researchers are also responsible for seeking ethical approval for their research if necessary (Prichard et al., 2015).

2 Aims

The specific aims of this project were:

1. To adapt and validate a fast and reliable analytical method for illicit drugs and pharmaceuticals in wastewater from Reykjavik.

2. To assess the use of illicit drugs and pharmaceuticals in Reykjavik by using WBE.

3. To compare results by WBE to other indicators of drug use.

4. To compare the estimated illicit drug use in Reykjavik to other Nordic countries found with WBE.

3 Materials and methods

3.1 Analytes of interest

The most commonly used illicit drugs in Iceland were selected for analysis along with pharmaceuticals with known abuse potential (Tyrfingsson, 2019). Illicit drugs and pharmaceuticals chosen for analysis in wastewater samples from Reykjavik are listed in Table 1.

Results on the analysis of wastewater samples from five Nordic capitals, Helsinki, Oslo, Reykjavik, Stockholm and Torshavn are presented in Paper IV (Löve et al., 2018). Illicit stimulants chosen for analysis in wastewater samples from the Nordic cities are listed in Table 2.

Table 1. Selected compounds for the analysis of wastewater from Reykjavik.

Parent compounds	Metabolites
Amphetamine	-
Cocaine	Benzoylecgonine
Codeine	-
MDMA	-
Methamphetamine	-
Methylphenidate	Ritalinic acid
Morphine	-
Tramadol	O-desmethyltramadol
-	THCA

Table 2. Selected compounds for the analysis of wastewater from five Nordic capitals.

Parent compounds	Metabolites
Amphetamine	-
MDMA	-
Methamphetamine	-
Cocaine	Benzoylecgonine
	Cocaethylene

3.2 Sample collection in Reykjavik

Wastewater enters the sewer system in Reykjavik from house connection such as residential homes or workplaces. The wastewater travels through the sewer system via lift/pump stations before entering WTPs. The maximum residence time of the wastewater in sewers is 3 hours (Sigurður Skarphéðinsson, Reykjavik Energy, oral communication). Treatment of wastewater in Reykjavik is limited compared to similar-size communities in the other Nordic countries. The two WTPs located in Reykjavik, Skerjafjarðarveita and Sundaveita, are designed to remove coarse material, fat, and sand, using filtration and sedimentation. Before the water is pumped into the recipient, 3 km out in Faxaflói-bay, sample collection and flow measurements take place. The flow of the wastewater stream is measured in a Parshall flume at a temporal resolution of 60 minutes. The design of the WTPs in Reykjavik is shown in Figure 9.

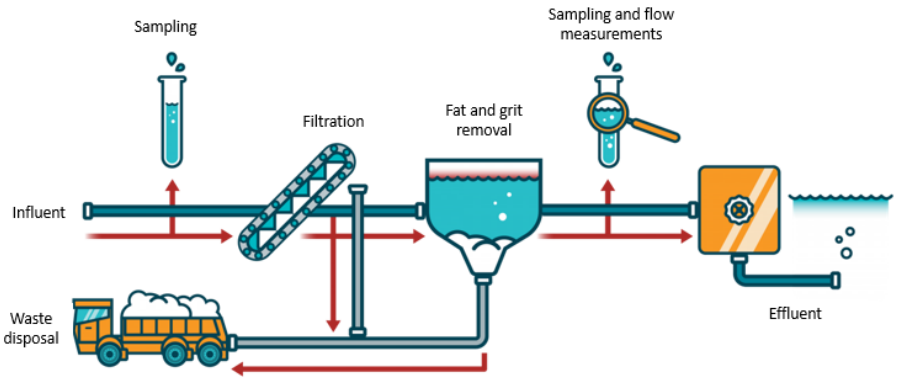

Figure 9. Scheme of the cleaning process of WTPs in Reykjavik. Published with permission from Veitur ohf.

Sample collection took place in the two WTPs located in Reykjavik, Skerjafjarðarveita and Sundaveita. A map of the catchment area is shown in Figure 10. Based on the population census, the residential population in this catchment area is approximately 180,000 individuals which is around 50% of the total population of Iceland and approximately 80% of the Reykjavik metropolitan area. Weeklong sampling periods at eleven different time points took place in 2017 (February, March, May, July, August, October, and November), 2018 (January and March), 2019 (April) and 2020 (June). Sample collection in 2019 and 2020 only took place in Sundaveita WTP. The collection of samples was performed using a WaterSam WS Porti automatic sampling device. This mobile sampling device is equipped with 12 glass bottles and a cooling system. Samples were collected in time-proportional sampling mode

where approximately 100 mL were collected at 80-minute intervals. Each sample was collected over 24 hours for seven days at a time. Samples were refrigerated at 4°C during sampling. The samples were then stored at -20°C immediately after sampling, without acidification, until sample preparation took place.

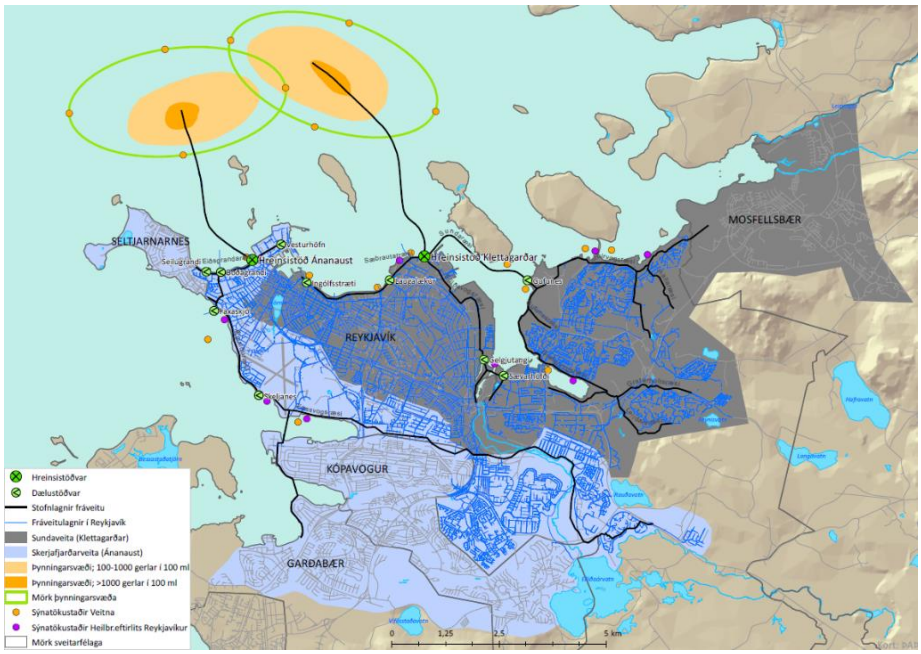

Figure 10. Map of Reykjavik showing the catchment area of the two WTPs, Skerjafjarðarveita (light blue area) and Sundaveita (dark blue area), and the recipient in Faxaflói-bay (yellow area).

3.3 Sample collection in five Nordic capitals

Sample collection took place in the spring and summer of 2016 in five Nordic capitals, Helsinki, Oslo, Reykjavik, Stockholm, and Torshavn. Samples were collected in Oslo over seven consecutive days, including four 24h samples during weekdays and a 3-day composite sample from Friday to Sunday. Sample collection in Stockholm took place in two WTPs on seven dispersed days, including five 24h samples during weekdays and two 24h samples during a weekend. Sample collection in Reykjavik, Helsinki and Torshavn took place over seven consecutive days. Samples were collected in two WTPs in Reykjavik. Detailed information on sample collection in the different countries are shown in Table 3.

Table 3. Sample collection details from five Nordic capitals (Löve et al., 2018).

City	Name of WTP	Number of inhabitants	Time of sampling	Flow range (m^3/24h)	Sampling mode
Helsinki	Viikinmäki	800000	March 2016	283800-353837	Volume proportional
Oslo	VEAS	607651	March 2016	250830 - 391527	Flow proportional
Reykjavik	Skerjafjarðarveita and Sundaveita	186000	August 2016	64725 - 73333	Time proportional
Stockholm	Henriksdal I and II	784000	May 2016	101500 - 136000	Flow proportional
Torshavn	Sersjantvíkin	820	April 2016	600 - 960	Time proportional

3.4 Pre-analytical challenges of THCA determination

The appropriate pre-treatment of samples is vital to prevent the degradation of analytes. In Paper I, methodological challenges related to the analysis of THCA in wastewater were investigated (Causanilles et al., 2017). This resulted from a scientific collaboration between 10 laboratories where several factors suspected to cause bias when analysing THCA were studied and a best-practice protocol was proposed. The following factors were included: freeze-thaw cycles, in-sample stability, filtration, and sorption to container surfaces.

Firstly, the effect of freeze-thaw cycles was investigated by spiking wastewater samples at 50 ng/mL and dividing them into aliquots. Each aliquot was frozen and thawed 0, 1, 2, 5, 10 and 20 times. Samples were analysed after all freeze-thaw cycles had been completed. Secondly, in-sample stability of THCA was tested over a 7-day period (time points at 0, 1, 4 and 7 days) and stored at three temperatures, 20°C, 4°C and -20°C. Samples were spiked at 50 ng/mL and distributed into three vials, each stored at different temperatures before analysis. Thirdly, the effect of sample filtration was tested at both neutral pH (pH~7.5) and acidic pH (pH=2.5). Wastewater samples spiked at 50 ng/mL were analysed after filtration with five different kinds of filters in various combinations. These filters were type GF/A glass microfiber filters, cellulose nitrate and acetate filters, type A/E glass fibre filters, PES membrane filters and regenerated cellulose filters. Recoveries of THCA were compared with spiked wastewater samples without filtration. Finally, sorption of THCA to container surfaces was assessed. Spiked samples at 50 ng/mL were placed in two different types of vials, glass, and polypropylene (PP). Both neutral (pH~7.5) and acidic (pH=2.5) conditions were tested. Samples were analysed after storage at 4°C after 0, 1, 4 and 7 days.

Based on results from the above-mentioned experiments, the order of preparatory steps performed before sample extraction was evaluated. These steps included the addition of internal standards, sample filtration, and acidification of samples. To determine the most appropriate order of steps, four different orders of sample preparation were tested. To confirm the best practice protocol based on these experiments, an inter-laboratory study was organized with eight laboratories. Wastewater samples were collected in Utrecht and used for this comparison. Four different test samples were prepared, non-spiked at neutral pH, spiked at high level (720 ng/L) at neutral pH, spiked at low level (72 ng/L) at neutral pH and finally spiked at high level (720 ng/L) at pH=2.5. The samples were frozen prior to being sent to each laboratory for analysis.

3.5 Pre-treatment of samples from Reykjavik

Considering the proposed order of preparatory steps for THCA in Paper I, it was decided to apply these conditions to all wastewater samples from Reykjavik before sample preparation and analysis of illicit drugs and pharmaceuticals. All samples were therefore kept at neutral pH after sample collection and kept frozen at -20°C until analysis. Corresponding internal standards were added to 50 mL of thawed wastewater samples at 180 ng/L. To avoid filtration, samples were centrifuged to remove solid particles. Sample preparation using SPE was performed on the supernatant before analysis with UPLC-MS/MS.

3.6 Pre-treatment of samples from five Nordic capitals

Wastewater samples from Oslo, Reykjavik, Stockholm, and Torshavn were shipped to the Norwegian Institute for Water Research (NIVA). All samples were kept at -20°C until analysis. Thawed samples were spiked with internal standards at a concentration of 500 ng/L prior to analysis. To avoid filtration and loss of compounds, samples were centrifuged, and the supernatant used for analysis. Wastewater samples from Helsinki were acidified with hydrochloric acid (pH=2) immediately after collection to prevent degradation. Prior to analysis, internal standards were added to samples from Helsinki and centrifuged to remove solid particulate matter (Kankaanpää et al., 2016, 2014).

3.7 Extraction of samples from Reykjavik

A sample extraction method was adapted based on Bijlsma et al. (2014), presented in Paper III. SPE was applied using 3 mL Waters Oasis HLB cartridges with 60 mg sorbent weight (Milford, MA, USA). The sample extraction procedure used for the analysis of illicit drugs and pharmaceuticals in wastewater from Reykjavik is shown in Figure 11.

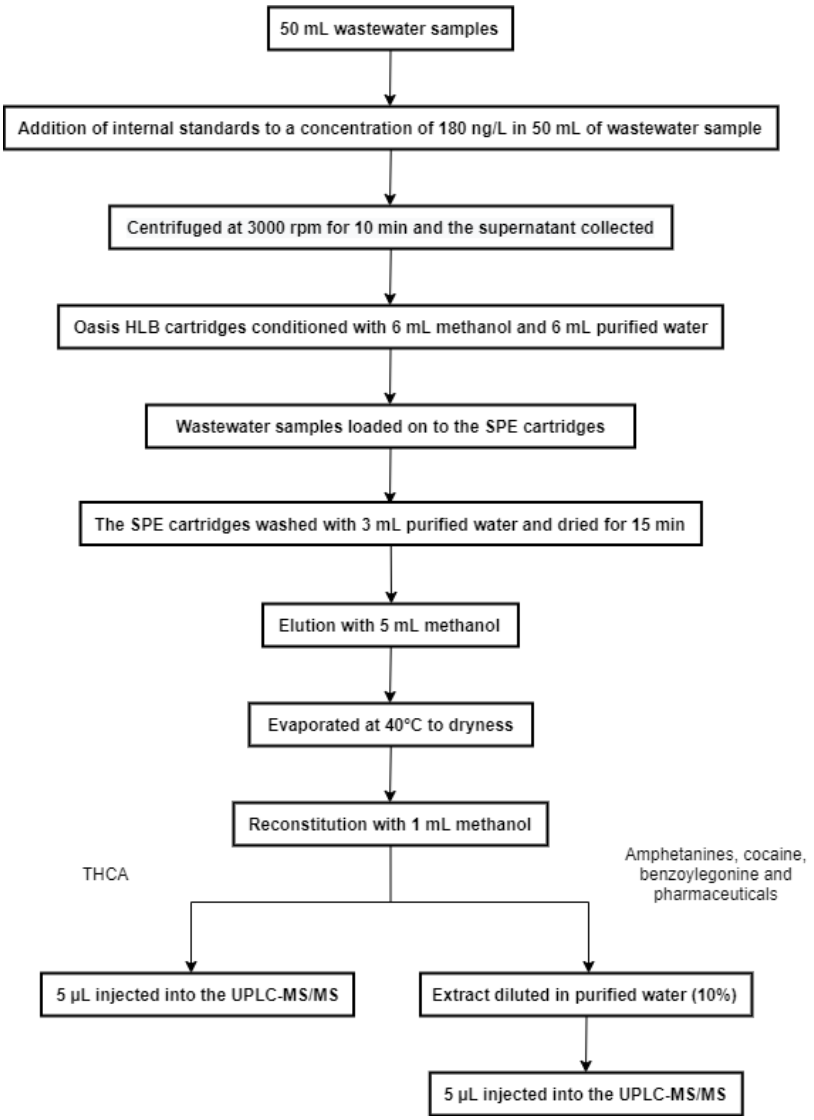

Figure 11. Graphical scheme of the sample extraction procedure used for wastewater samples from Reykjavik.

3.8 Extraction of samples from five Nordic cities

A SPE micro-extraction method combined with large volume injection and post-loop mixing is presented in Paper II, Wastewater samples from Oslo, Reykjavik, Stockholm, and Torshavn were analysed according to this extraction method and the results presented in Paper IV. SPE was applied using Waters Oasis HLB µElution plates (Milford, MA, USA). The sample extraction procedure is shown in Figure 12. Preparation of samples from Helsinki was according to Kankaanpää et al. and was performed at the National Institute for Health and Welfare in Helsinki (Kankaanpää et al., 2016, 2014).

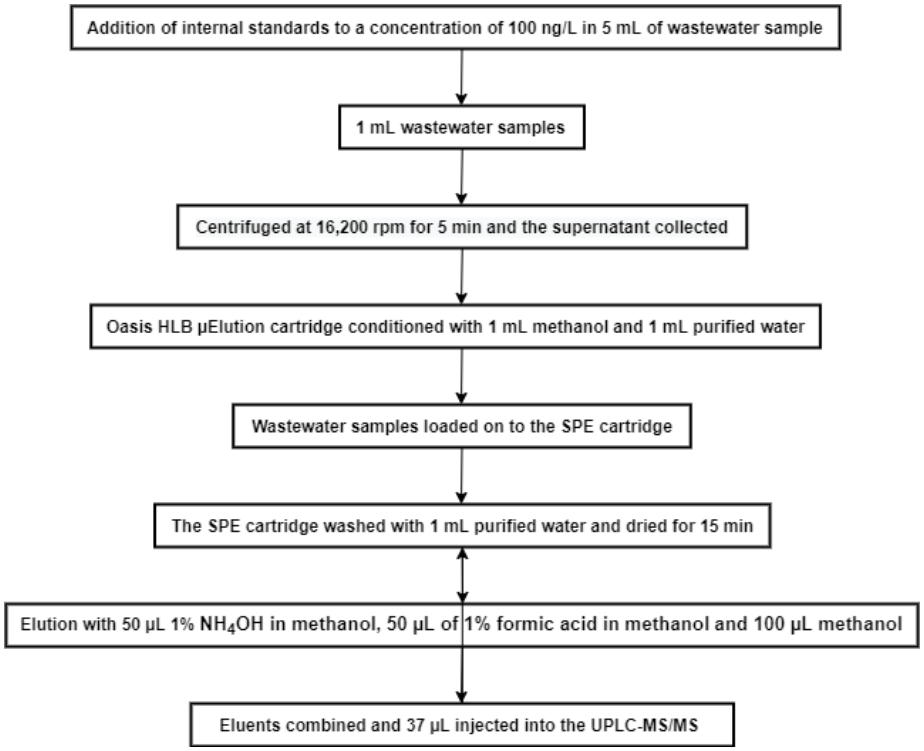

Figure 12. Graphical scheme of the SPE micro-extraction procedure used for wastewater samples from four Nordic capitals.

3.9 Analysis of samples from Reykjavik

Instrumental analysis of wastewater samples from Reykjavik (presented in Paper III) was performed using a Waters Acquity UPLC I-Class system coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer with a ESI source (Milford, MA, USA). The instrumental method was adapted from an existing in-house method by infusing analytes of interest and optimizing their different parameters. Selected quantifier and qualifier transitions along with other MS/MS parameters for each compound are presented in Table 4.

Table 4. Selected quantifier and qualifier transitions along with other MS/MS parameters used in the analysis of wastewater samples from Reykjavik.

Compound	ESI polarity	Quantifier transition (Q1 > Q3)	Cone / Collision (V / V)	Qualifier transition (Q1 > Q3)	Cone / Collision (V / V)
Amphetamine	+	136 > 119	25 / 15	136 > 90.9	25 / 7
Amphetamine-d3	+	139 > 92.0	25 / 15	-	-
Methamphetamine	+	150 > 119	35 / 16	150 > 90.9	35 / 10
Methamphetamine-d5	+	155 > 92.0	27 / 15	-	-
MDMA	+	194 > 163	36 / 23	194 > 105	36 / 11
MDMA-d5	+	199 > 165	32 / 10	-	-
Cocaine	+	304 > 82.0	38 / 28	304 > 182	38 / 18
Cocaine-d3	+	307 > 185	38 / 18	-	-
Benzoylecgonine	+	290 > 168	45 / 27	290 > 82.0	45 / 18
Benzoylecgonine-d3	+	293 > 171	38 / 18	-	-
THCA	-	343 > 245	60 / 26	343 > 299	60 / 18
THCA-d3	-	346 > 302	60 / 18	-	-
Methylphenidate	+	234 > 84.0	40 / 42	234 > 56.0	40 / 20
Ritalinic acid	+	220 > 56.0	35 / 43	221 > 84.0	35 / 20
Ritalinic acid-d5	+	225 > 84.1	20 / 18	-	-
Codeine	+	300 > 215	50 / 40	300 > 165	50 / 24
Codeine-d3	+	303 > 215	50 / 24	-	-
Morphine	+	286 > 201	60 / 39	286 > 153	60 / 23
Morphine-d3	+	289 > 201	45 / 24	-	-
Tramdol	+	264 > 58.0	37 / 10	264 > 246	37 / 14
Tramadol-d3	+	268 > 58.1	26 / 19	-	-
O-desmethyltramadol	+	250 > 58.0	37 / 14	250 > 232	37 / 10

A Waters Acquity UPLC® BEH C18 column, 2.1×100 mm, with a particle size of 1.7 µm (Milford, MA, USA) was used for chromatographic separation. The column was kept at a temperature of 40°C during analysis. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) with a flow rate of 0.4 mL/min. The gradient of the mobile phase was as follows: 2% B from 0 to

1.5 min, 13% B at 1.8 min, 36% B at 2.65 min, 50% B at 3.4 min, 95% B at 3.45 min, 2% B at 2.8 min. The injection volume was 5 µL.

Instrumental conditions for the mass spectrometer were as follows: the flow rate of the cone gas and desolvation gas was 20 L/h and 800 L/h, respectively. The cone gas was nitrogen and collision gas argon. Capillary voltage was 1 kV. The source temperature and desolvation temperature was 150°C and 500°C, respectively. Data acquisition was performed using multiple reaction monitoring (MRM).

3.9.1 Method validation

The analytical method presented in Paper III was validated according to published guidelines by the European Medicines Agency (EMA), the International Union of Pure, Applied Chemistry (IUPAC) and the Scientific Working Group for Forensic Toxicology (SWGTOX) with appropriate modifications (European Medicines Agency, 2011; International Union of Pure and Applied Chemistry, 2002; Kruve et al., 2015a, 2015b; Scientific Working Group for Forensic Toxicology, 2013). The acceptability of method performance and reliability of results was evaluated by testing the following: linearity, limit of quantification, accuracy, precision, recovery, and matrix effects.

The linear relationship between the response of the instrument and concentrations of analytes was evaluated according to guidelines published by IUPAC (International Narcotics Control Board, 2011). The validated limit of quantification (LOQ) was 5 ng/L for all analytes. A calibration curve was constructed containing 10 calibration standards with analyte concentrations between 0.1 ng/mL and 30 ng/mL, prepared in methanol in duplicate. The regression coefficient (R^2) and a residual plot was used to determine if the calibration curve was linear. A regression coefficient > 0.99 and randomly distributed residuals indicated satisfactory linearity.

Both intra-day and inter-day precision was assessed according to guidelines published by EMA and SWGTOX (European Medicines Agency, 2011; Scientific Working Group for Forensic Toxicology, 2013). Quality control samples were prepared by spiking tap water samples at three concentration levels, 5 ng/L, 15 ng/L and 250 ng/L in five replicates (n=5). These samples were prepared using other stock solutions of external standards than were used for the calibration curve, as recommended by the EMA (European Medicines Agency, 2011). The run was repeated three times and analysed on

different days to estimate inter-day precision. A coefficient of variation (CV) below 15% for the medium and high concentrations, and below 20% for the low concentration was considered satisfactory.

Accuracy of the method was estimated according to the EMA guideline (European Medicines Agency, 2011). Tap water samples were spiked at three concentration levels (5 ng/L, 15 ng/L and 250 ng/L) in five replicates each (n=5). The experiment was repeated on three different days. For the medium and high concentrations, the accuracy was considered satisfactory if the mean concentration was within 15% of the theoretical concentration. For the low concentration, satisfactory accuracy was within 20%.

Matrix effects and extraction recoveries were evaluated according to Matuszewski et al. (2003). Three sample sets were prepared. Sample set 1 consisted of standard solutions in methanol spiked at 7.5 ng/mL. Sample set 2 consisted of the residue of extracted tap water spiked at 7.5 ng/mL. Sample set 3 was prepared by spiking tap water before extraction at 150 ng/L. Recoveries were evaluated as the CV between peak areas of analytes in sample sets 2 and 3. Matrix effects were calculated as the CV between peak areas of analytes in sample sets 2 and 1. Satisfactory matrix effects did not exceed 15% CV (European Medicines Agency, 2011).

3.10 Analysis of samples from five Nordic cities

Instrumental analysis of wastewater samples from Oslo, Reykjavik, Stockholm, and Torshavn was performed using a Waters Acquity UPLC system coupled to a Waters Quattro Premier XE Micromass triple quadrupole mass spectrometer (Milford, MA, USA). This instrument was equipped with a T-wave collision cell and ESI source. Detailed description of instrumental parameters can be found in Paper II. Instrumental analysis of wastewater samples from Helsinki was performed according Kankaanpää et al. at the National Institute for Health and Welfare in Helsinki (Kankaanpää et al., 2016, 2014).

A Waters Acquity UPLC® BEH C8 column, 2.1×100 mm, with a particle size of 1.7 µm (Milford, MA, USA) was used for chromatographic separation. The column was kept at a temperature of 50°C during analysis. The mobile phase consisted of 0.1% ammonium hydroxide (A) and acetonitrile (B) with a flow rate of 0.4 mL/min. The gradient of the mobile phase was as follows: 3% B from 0 to 4.9 min, 40% B at 5.1 min, 60% B at 8.5 min, 95% B at 9 to 10 min, 3% B at 10.5 to 11 min. Large volume injection combined with post-loop mixing was used. The injection volume was 37 µL.

Instrumental conditions for the mass spectrometer were as follows: the flow rate of the cone gas and desolvation gas was 50 L/h and 800 L/h, respectively. The collision gas was argon with a flow rate of 0.15 mL/min. Capillary voltage was 3.2 kV. The source temperature and desolvation temperature was 100°C and 450°C, respectively. Data acquisition was performed using MRM. Selected quantifier and qualifier transitions along with other MS/MS parameters for each compound are shown in Table 5.

Table 5. Selected quantifier and qualifier transitions along with other MS/MS parameters used in the analysis of wastewater samples from four Nordic capitals.

Compound	ESI polarity	Quantifier transition (Q1 > Q3)	Qualifier transition (Q1 > Q3)	Cone / Collision (V / V)
Amphetamine	+	136.1 > 91.1	136.1 > 119.1	20 / 15
Amphetamine-d8	+	144.1 > 97.1	-	20 / 15
Methamphetamine	+	150.1 > 91.1	150.1 > 119.1	20 / 15
Methamphetamine-d11	+	161.2 > 127.1	-	20 / 15
MDMA	+	194.2 > 163.2	194.2 > 105.1	20 / 15
MDMA-d5	+	199.2 > 165.2	-	20 / 15
Cocaine	+	304.2 > 182.2	304.2 > 105.0	30 / 20
Cocaine-d3	+	307.2 > 185.2	-	30 / 22
Benzoylecgonine	+	290.2 > 168.2	290.2 > 105.0	30 / 20
Benzoylecgonine-d3	+	293.2 > 171.2	-	30 / 20
Cocaethylene	+	318.2 > 196.2	318.2 > 82.1	30 / 20
Cocaethylene-d3	+	321.2 > 199.1	-	30 / 20

3.10.1 Method validation

Analytical methods presented in Paper II were validated according to guidelines published by Eurachem with minor modifications (Eurachem, 2014).

The linearity was evaluated by constructing a calibration curve containing eight concentrations between 0.025 ng/mL and 10 ng/mL, in methanol in triplicate. A regression coefficient > 0.99 indicated satisfactory linearity. LOQ was calculated for all analytes as the concentration giving a signal to noise ratio higher than 10.

Recoveries were tested by spiking wastewater samples at 100 ng/L in triplicate along with the internal standards prior to sample extraction. Recoveries between 80% and 120% were considered acceptable. Intra-day precision was evaluated by spiking six wastewater samples at 200 ng/L and

the results were expressed by calculating the relative standard deviation (RSD) between samples.

Matrix effects were assessed by spiking wastewater samples at 1 ng/mL in triplicate with both analytes and internal standards. Responses in non-spiked wastewater samples were subtracted from the responses in spiked wastewater samples. Results were compared with analyte responses in the mobile phase at the same concentration.

3.11 Back-calculations

Back-calculated consumed amounts of illicit drugs based on their concentrations analysed in wastewater from Reykjavik are presented in Paper III. The equation used for back-calculations of population normalized mass loads of analytes in wastewater (mg/day/1000 inhabitants) is shown in Figure 13. The daily mass loads were calculated by multiplying the concentrations of the compounds of interest (mg/L) analysed in each wastewater sample with the flow rate of the wastewater stream (L/day). To account for urinary excretion patterns of each analyte, the mass loads (mg/day) were then multiplied by a correction factor to obtain the consumed amounts (mg/day). The correction factor is based on the molecular mass ratio of each analyte divided by the excretion percentage. Correction factors used in back-calculations and parameters used to obtain them are listed in Table 6. The daily mass loads were then normalized by the population size of the catchment area based on census data to achieve the consumed amounts for 1000 inhabitants (mg/day/1000 inhabitants) (Statistics Iceland, 2020). The number of inhabitants for each year of sampling (2017 to 2020) are listed in Table 7.

$$\frac{mg/day}{1000\ inhabitants} = \left(\frac{CONC\ (mg/L) \times\ FR\ (L/day)\ \times\ CF}{POP}\right) \div 1000$$

Figure 13. Equation for back-calculations of population normalized mass loads of analytes in wastewater. CONC=measured concentration of each analyte in wastewater (mg/L), FR=average flow rate of the watewater stream (L/day), POP=number of inhabitants in the catchment area, CF=correction factor.

In Paper IV, the aim was to achieve a reliable comparison between countries and therefore it was not necessary to back-calculate concentrations to consumed amounts. Comparison was achieved by using daily mass loads (ng/day) of the stimulant drugs amphetamine, methamphetamine, MDMA and cocaine in the wastewater. This was performed by multiplying the analysed concentrations in wastewater (ng/L) by the daily average flow rate (L/day). Results were population normalized to obtain the daily mass loads of each analyte in mg/day/1000 inhabitants.

Table 6. Correction factors for each analyte used in the back-calculations of concentrations in wastewater from Reykjavik and parameters used to obtain them.

Drug target residue	Mean excretion (%)	Route of administration	Molecular mass ratio	Correction factor
Illicit drugs				
Amphetamine	36.1[a]	Oral	1.00	2.77
Methamphetamine	39.3[a]	Intranasal	1.00	2.54
MDMA	22.5[a]	Oral	1.00	4.40
Benzoylecgonine	29.2[b]	*	1.05	3.59
THCA	0.500[a]	Smoked	0.910	182
Pharmaceuticals				
Methylphenidate	2.00[c]	Oral	1.00	50.0
Ritalinic acid	60.0-81.0[d]	Oral	1.06	1.52
Morphine	87.0[d]	Oral	1.00	1.15
Codeine	70.0[e]	Oral	1.00	1.40
Tramadol	29.0[d]	Oral	1.00	3.44
O-desmethyltramadol	20.0[d]	Oral	1.05	5.25

[a](Gracia-Lor et al., 2016)
[b](Castiglioni et al., 2013)
[c](Baz-Lomba J.A. et al., 2016)
[d](Baselt, 2017)
[e](Terzic et al., 2010)
*Weighted mean of excretion percentages for different routes of administrations

Table 7. Number of inhabitants behind Skerjafjarðarveita and Sundaveita WTPs during each year of sample collection in Reykjavik, based on census data.

Year of census	Sundaveita WTP Number of inhabitants	Skerjafjarðarveita WTP Number of inhabitants
2017	97000	87000
2018	99000	89000
2019	102000	-
2020	103000	-

3.12 Inter-laboratory comparison

Since 2015, participation in inter-laboratory comparison studies organized by the European-wide network SCORE has ensured the quality of measurements performed on wastewater from Reykjavik.

Two different types of test samples were shipped to each laboratory to estimate its analytical performance. Firstly, a methanol solution containing unknown concentrations of analytes. Secondly, three different tap water samples spiked with unknown concentrations of analytes (in low, medium, and high concentrations). Thirdly, a blank tap water sample. Target analytes were cocaine, benzoylecgonine, amphetamine, methamphetamine, MDMA and THCA. Each laboratory was asked to deliver results for five replicates of each methanol sample and three replicates of the tap water samples. Samples were shipped frozen to each laboratory for analysis. Z-scores were used to evaluate the performance of each laboratory and were calculated according to the group's mean. Outliers were excluded using a Grubbs' test (van Nuijs et al., 2018).

3.13 Comparison with other indicators of drug use

In Paper III, results by WBE were compared with data on DUI cases and seized amounts of drugs. Data on the number of DUI cases was obtained from the Department of Pharmacology and Toxicology, University of Iceland. This data included both negative and positive DUI samples from 2014 to 2020. Requested analysis by the police is based on preliminary testing of cannabis, cocaine, and amphetamines. Amphetamines are analysed as a package and include MDMA, methamphetamine and amphetamine.

The National Commissioner of the Icelandic Police provided data on seized amounts of illicit drugs from 2006 to 2019. This data contained information on seized amounts of amphetamine, methamphetamine, and cocaine in powder form along with data on seized amounts of amphetamine base intended for production. Data on seized amounts of MDMA contained both information in grams and pieces (tablets). Data on seized amounts of MDMA in pieces was therefore transformed into grams as follows: data on the average weight (mg) of tablets received for analysis from 2010 to 2019 was obtained from the Department of Pharmacology and Toxicology along with the average concentration of MDMA crystals from 2012 to 2019. The conversion to grams was achieved by multiplying the average weight of tablets by the number of seized tablets, divided by the average concentration of MDMA crystals. Data on seized amounts of cannabis contained the number of plants in active soil cultivation, grams of plants in the drying process, grams of marijuana ready to be sold on the drug market and grams of hash.

Results on the use of pharmaceuticals by WBE are presented in this thesis. These results were compared with data from the Icelandic Prescription Medicines Register. The data contained the number of DDDs of filled prescriptions of morphine, codeine, tramadol, and methylphenidate in the Reykjavik metropolitan area from 2017 to 2019. The area was selected by postal codes and corresponded with the catchment area where wastewater sampling took place.

4 Results and discussion

4.1 Method adaptation

No previous experience in the analysis of illicit drugs and pharmaceuticals in wastewater was available in Iceland in the beginning of this project as well as no published data where WBE had been applied. The primary aim of this project was therefore to adapt a reliable analytical method for the quantification of drugs of abuse in wastewater.

Wastewater samples are complex matrixes that need extensive cleaning to effectively remove components that can interfere with the analytes of interest. Residential water rates in Iceland are low and water is accessible to all inhabitants in abundant amounts (Árnadóttir et al., 2020). High water use could cause the dilution of the wastewater resulting in low concentrations of analytes. A pre-concentration step with high sample volumes were therefore chosen for the analysis of drug residues in wastewater from Reykjavik along with sensitive analytical equipment (van Nuijs et al., 2011a). SPE is most commonly described in the literature as an appropriate sample extraction method for illicit drugs and pharmaceuticals in wastewater (van Nuijs et al., 2011a).

Method adaptation was performed according to Bijlsma et al. (2014) who presented a SPE method for the simultaneous analysis of various illicit drugs and pharmaceuticals. Selected analytes according to Tables 1 and 4 are all included in the analytical method by Bijlsma et al. (2014) except for tramadol and its metabolite O-desmethyltramadol. The method showed acceptable recoveries (between 70% and 120%) and was therefore considered an appropriate option for the adaptation for wastewater samples from Reykjavik.

4.2 Method validation

In Paper III, an analytical method is presented for the simultaneous analysis of commonly used illicit drugs in Iceland. This method was validated for both illicit drugs and pharmaceuticals according to published guidelines (European Medicines Agency, 2011; International Union of Pure and Applied Chemistry, 2002; Scientific Working Group for Forensic Toxicology, 2013).

The linearity of calibration curves for all analytes met requirements of linear regression $R^2 > 0.99$. Regression coefficients for all analytes are listed in Table 9. Residual plots were also constructed which showed acceptable distribution (Scientific Working Group for Forensic Toxicology, 2013). Based on preliminary testing on concentrations of analytes in Reykjavik wastewater it was considered unnecessary to lower the LOQ further.

Validation results for accuracy, within-day precision and intermediate precision experiments met the criteria (European Medicines Agency, 2011; Scientific Working Group for Forensic Toxicology, 2013). Accuracy of the method is presented as bias (%) between the analysed concentration (n=5) and theoretical value. The bias did not exceed 20% for the LOQ (5 ng/L) and 15% for the medium and high concentrations (15 ng/L and 250 ng/L). The precision of the method is presented as CV (%) and was ≤ 15% for the medium and high concentrations, and ≤ 20% for the low concentration. These results are considered satisfactory according to the above-mentioned guidelines. The results for accuracy and precision of the method are shown in Table 8.

Table 8. Validation results for accuracy (%Bias), within-day precision and intermediate precision (%CV) at three concentrations for all analytes of interest.

Compound	Accuracy (%Bias)			Within-day precision (%CV)			Intermediate precision (%CV)		
	5 ng/L	15 ng/L	250 ng/L	5 ng/L	15 ng/L	250 ng/L	5 ng/L	15 ng/L	250 ng/L
Amphetamine	6.0	7.5	1.6	8.1	3.1	3.6	20	6.7	4.0
Benzoylecgonine	10	2.1	1.2	10	2.3	1.1	9.8	6.0	2.0
Cocaine	2.0	7.2	0.61	17	4.6	1.3	19	9.9	2.5
Codeine	20	8.5	0.0	13	13	4.0	13	9.7	3.9
Methamphetamine	1.8	7.8	3.9	14	12	1.8	17	9.9	3.2
Methylphenidate	14	6.5	7.3	8.2	6.6	9.8	13	11	8.7
MDMA	3.8	3.2	2.2	11	3.9	1.4	14	9.3	1.9
Morphine	5.2	1.3	3.5	7.5	7.3	2.7	12	7.1	2.3
O-desmethyltramadol	7.4	7.4	16	7.1	7.4	9.1	14	7.1	10
Ritalinic acid	4.2	8.7	3.7	8.1	3.5	3.2	12	11	6.7
THCA	2.0	2.1	1.2	9.0	2.8	0.56	5.9	7.1	1.1
Tramadol	0.8	2.9	2.2	15	8.3	1.7	13	6.3	2.2

Results from the estimation of matrix effects are shown in Table 9. According to the criteria, acceptable matrix effects should not exceed 100 ± 15% CV (European Medicines Agency, 2011; Matuszewski et al., 2003).

Results showed that matrix effects ranged between 68.7% and 117%. Ten compounds out of twelve in total were within the criteria. Results for amphetamine showed 117% matrix effects and THCA showed 68.7% matrix effects. These results indicate a slight ion enhancement for amphetamine and some ion suppression for THCA. Corresponding internal standards were used for the analysis of these compounds which compensated for the estimated matrix effects which was confirmed with inter-laboratory testing. These results were therefore considered to be acceptable.

Table 9. Validation results for matrix effects (%), recoveries (%) and linearity (R^2) for all analytes of interest at 150 ng/L.

Compound	Matrix effects (% ± SD)	Recovery (% ± SD)	Regression coefficients (R^2)
Amphetamine	117 ± 12	113 ± 25	0.9994
Benzoylecgonine	97.1 ± 7.3	98.0 ± 2.6	0.9998
Cocaine	115 ± 26	102 ± 4.0	0.9995
Codeine	109 ± 13	95.4 ± 3.0	0.9994
Methamphetamine	109 ± 9.4	95.1 ± 7.2	0.9987
Methylphenidate	114 ± 27	80.4 ± 29	0.9997
MDMA	111 ± 11	93.7 ± 2.0	0.9989
Morphine	113 ± 7.1	98.3 ± 3.1	0.9996
O-desmethyltramadol	107 ± 4.7	170 ± 10	0.9998
Ritalinic acid	94.7 ± 6.4	138 ± 14	0.9990
THCA	68.7 ± 4.9	102 ± 3.6	0.9980
Tramadol	110 ± 9.9	99.2 ± 1.7	0.9992

Recoveries of analytes are shown in Table 9. The recoveries of analytes were above 80% in all cases. The recovery of amphetamine was 113%. High variability between samples (25% SD) could point to contamination in some of the samples. There was a considerable risk of contamination due to the handling of seized illicit drugs in the same laboratory. Preventative measures were taken to minimize these effects as much as possible by avoiding simultaneous evaporation of wastewater samples with other samples as well as rinsing all glassware with methanol before use. The recovery of ritalinic acid was 138%. Extremely high recoveries could indicate that further method development is needed where the more optimal way could be to extract ritalinic acid and methylphenidate from wastewater separately from other analytes.

Oasis MCX cartridges have been used for the extraction of ritalinic acid in wastewater giving better recoveries (Burgard et al., 2013). Therefore, using Oasis MCX cartridges as opposed to Oasis HLB could be a possible solution for better recoveries of these compounds. The recovery for O-desmethyltramadol was 170%. A corresponding internal standard was not available for O-desmethyltramadol and therefore tramadol-d3 was utilized. This lack of a corresponding internal standard likely explains this outlier. It is therefore recommended to use corresponding internal standards for all compounds during future analysis.

In-house controls were analysed with all samples (tap water spiked at 40 ng/L and 150 ng/L) to further confirm the accuracy of the method and avoid overestimation due to contamination. All runs where the concentrations of analytes in the in-house controls exceeded 20% from the theoretical concentrations were repeated after a requirement analysis had been performed.

4.3 Inter-laboratory testing

A very good indication of the reliability of results is participation in inter-laboratory testing. This was conducted yearly from 2015 to 2019 under the auspices of the SCORE collaboration (SCORE, 2020). Commonly used illicit drugs were tested including amphetamine, methamphetamine, MDMA, cocaine, and THCA. If the inter-laboratory tests showed satisfactory results, z-scores < 2.0 from the group mean, results for stimulant drugs based on a weeklong sampling period were published by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (SCORE, 2020). Results for THCA are currently not published by the EMCDDA due to its known pre-analytical challenges addressed in Paper I. When analysing test samples from 2015 to 2017, amphetamine-d3 was used as an internal standard for amphetamine, methamphetamine and MDMA. This resulted in z-scores > 2.0 for MDMA and methamphetamine causing results for MDMA in 2015 and methamphetamine in 2017 not to be published by the EMCDDA (SCORE, 2020). Corresponding internal standards for both methamphetamine and MDMA were used in 2018 and 2019 with very acceptable results. Further, all wastewater samples collected in Reykjavik from 2017 to 2020 were analysed using corresponding internal standards for all analytes.

4.4 Pre-analytical challenges

Analytical challenges in the determination of THCA such as ionization suppression due to matrix effects and poor sensitivity have previously been reported (Bijlsma et al., 2014; Hernández et al., 2018; Ort et al., 2014). Factors related to the pre-treatment of samples such as adsorption to solid particulate matter and stability of compounds have also been suggested to affect the analysis of THCA (Hernández et al., 2018; McCall et al., 2016). These challenges have been associated with the physio-chemical properties of THCA which differ from other illicit drugs and their metabolites in its low polarity (Causanilles et al., 2017). Results from inter-laboratory testing supported these analytical problems with deviations up to 90% in spiked tap water compared to deviations under 25% in methanol samples (van Nuijs et al., 2018).

In Paper I, sources of bias related to the analysis of THCA were identified with a collaborating effort of ten laboratories in Europe (Causanilles et al., 2017). Several parameters that were suspected to affect the recovery of THCA were tested. Tested parameters were freeze-thaw cycles, in-sample stability, filtration, and sorption to different container surfaces. The acidification of samples to pH=2 has been reported to stabilise some compounds in wastewater samples but also indicated the loss of THCA during sample pre-treatment (Kankaanpää et al., 2014; Senta et al., 2014). Therefore, pH adjustment was also included as an experimental parameter.

Testing of in-sample stability took place over seven days at three temperatures (20°C, 4°C and -20°C). Results showed that THCA remained stable during that time at all temperatures. The high stability of THCA for up to 4 months had been reported in previous studies (Heuett et al., 2015). However, decreased stability of THCA by 54% in acidified samples (pH=2) compared to non-acidified samples had also previously been reported (Senta et al., 2014). This could be explained by increased adsorption of THCA to solid particulate matter present in the wastewater sample at low pH compared to neutral pH.

When the effects of different filtration steps were tested, the results showed that more than 75% loss of THCA was observed at low pH (pH=2.5) demonstrating that THCA is highly pH dependent where the compound is both uncharged and in its hydrophobic form. The results therefore showed that the filtration step has great impact on the recovery of THCA. At neutral pH when large volumes of wastewater were filtered, significant losses of 27-30% were also observed.

Sorption of THCA to the surface of the sample container was tested and further showed the negative impact of low pH on the recovery of THCA. Three laboratories also provided results on the effect of freeze-thaw cycles. The effect of multiple freeze-thaw cycles on the recovery of THCA was not significant (≤10%).

Three of the four parameters tested, filtration, in-sample stability, and sorption experiments, were found to be pH dependant, showing decreased recoveries of THCA at low pH. According to these results, a best practice protocol was proposed. The recommended order of sample preparation steps was as follows: firstly, the addition of internal standards, then the filtration of samples followed by pH adjustment to acidic conditions (only if necessary). To confirm that this order of sample preparation steps was satisfactory when it comes to THCA recovery, an inter-laboratory study was performed. Z-scores were all within acceptable criteria (z-score ≤ 3) and recoveries for THCA were satisfactory, ranging from 64% to 112%. Recovery for acidified samples was only 54% which showed the negative impact of low pH on THCA recovery. The results from the inter-laboratory study therefore confirmed the best-practice protocol proposed. It was decided to use the recommended protocol for the analysis of samples from Reykjavik (described in Paper III). All samples from Reykjavik were kept at neutral pH and centrifuged before sample extraction to prevent loss of all compounds including THCA.

4.5 SPE micro-extraction

Sample preparation methods used for the extraction of illicit drugs and pharmaceuticals in wastewater are most commonly SPE methods where large volumes of sample (50 mL to 1000 mL) are necessary to reach the desired detection limits (Baz-Lomba et al., 2018; van Nuijs et al., 2011a). These methods are extremely time consuming and labour intensive.

In Paper II, a solid phase micro-extraction method was proposed. Smaller sample volumes of only 1 mL were extracted using 96 well SPE plates with reduced amounts of column sorbent. By using this method, it is possible to perform sample extraction on a much higher number of samples in a shorter amount of time compared to conventional SPE methods. To reach the necessary method sensitivity, large volume injection was used. The large volume injection is based on increased volumes of sample being injected into the UPLC system (37 µL) compared to more conventional injection volumes of e.g. 2-5 µL (Baz-Lomba et al., 2018; Boix et al., 2015). Highly selective

instruments such as the UPLC system are required to reach desired detection levels. Nevertheless, the small particles packed in the UPLC columns causes them to have lower sample capacity when using large volume injection. This can lead to chromatographic problems such as peak broadening and volume overload. Combining large volume injection with post-loop mixing has been shown to prevent these issues (Baz-Lomba et al., 2018). According to this configuration the organic phase is directed straight to the loop in the autosampler where it meets the sample (also diluted in organic phase). In the mixer, the sample is completely diluted in the aqueous phase and is carried to the UPLC column. In order to retain the sample at the head of the column, the aqueous phase is kept at a high ratio (97%) for the first 5 min. This causes the sample to dilute completely in the aqueous phase before it reaches the UPLC column. The post-loop mixing configuration is shown in Figure 14. By combining the micro-extraction with the large volume injection and post-loop mixing, acceptable chromatographic separation and detection limits can be achieved. This also increases the throughput of the method by minimizing sample volumes and simplifying the extraction procedure (Baz-Lomba et al., 2018).

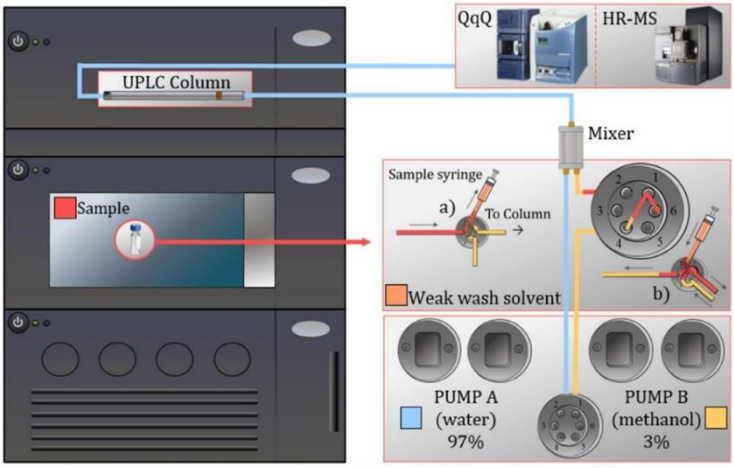

Figure 14. Configuration of the post-loop mixing process (Baz-Lomba et al., 2018).

The performance of the method was tested for cocaine, benzoylecgonine, amphetamine, methamphetamine, and MDMA by participating in an inter-laboratory comparison study (SCORE, 2020). The results were satisfactory for the studied analytes with scores of $|z| < 2$. Further, the method showed good linearity for all analytes with a $R^2 \geq 0.99$. Both absolute and relative recoveries

were tested for the micro-extraction method. Absolute recoveries were above 79% for all analytes except amphetamine which had a recovery of 36%. Relative recoveries were between 92% and 110% and were considered satisfactory. The precision of the method also met requirements with RSD between 3.4% and 14.4%. Only moderate matrix effects were observed for most compounds (did not exceed ± 20%) and were acceptable for all compounds.

The main advantages of SPE micro-extraction for wastewater samples is the high sample throughput. Up to 96 samples can be extracted at a time which reduces the labour intensity considerably. By using less sample volume, the amounts of internal standards and solvents also reduce substantially. Overall, the final cost of each sample is thus reduced. The combination of large volume injection and post-loop mixing further simplifies the sample extraction procedure by avoiding the reconstitution of the sample in the aqueous phase. The method efficiency is also improved by using the large volume injection, injecting 37 µL out of 100 µL as opposed to e.g. 2-5 µL out of 250-1000 µL presented in previously published methods. Considering the many advantages of the SPE micro-extraction combined with large volume injection and post-loop mixing, the method would be extremely feasible for further analysis of Icelandic wastewater samples. The large volume injection involves some adjustments to the available equipment and has therefore not yet been adapted.

4.6 Temporal trends in illicit drug use in Reykjavik

Results on illicit drug use in Reykjavik by WBE are presented in Paper III. Temporal trends were examined during eleven weeklong sampling periods between 2017 and 2020.

Amphetamine use was fairly stable from 2017 to 2018 but had increased significantly by 60% in April 2019 compared to March 2018 ($p < 0.05$, t-test). Amphetamine use in June 2020 during the COVID-19 pandemic was similar to April 2019. Although clear signs of increased amphetamine use are shown, the extensiveness of this rise cannot be confirmed as only two weeklong sample collections were performed in 2019 and 2020. This rise could therefore be explained by temporal variability. It is also not possible to distinguish if increasing amounts in wastewater can be traced back to a rising number of users, rise in consumed doses or increased purity of drugs (Bruno et al., 2018). Amphetamine supply in Iceland is estimated to have been stable in recent

years where organized crime groups are largely considered to dominate import and production of the drug (National Police Commissioner of Iceland, 2019). However, the average purity of amphetamine in Europe has increased over the past decade (European Monitoring Centre for Drugs and Drug Addiction, 2020). Increased purity of amphetamine on the Icelandic drug market could therefore partly explain the rise in amphetamine levels in wastewater.

Methamphetamine use estimated by WBE increased significantly by 125% from February 2017 to January 2018 ($p < 0.05$, t-test) but had decreased approximately by 20% in March 2018. The results showed stable use from March 2018 to June 2020. These results indicate that the COVID-19 pandemic had not affected the use of methamphetamine in Reykjavik at that timepoint. Overall, the results seem to indicate increasing use of methamphetamine in Reykjavik although the variability between sampling periods could point to some fluctuations in the availability of the drug. These results are in accordance with reports on rising availability of methamphetamine in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2020). Nevertheless, estimated methamphetamine use was very low compared to amphetamine, which is in agreement with the European drug market where amphetamine is more commonly available compared to methamphetamine (European Monitoring Centre for Drugs and Drug Addiction, 2020; González-Mariño et al., 2020).

MDMA use by WBE remained stable from 2017 to 2020. These results indicate that the COVID-19 pandemic had not affected the use of MDMA in Reykjavik at that timepoint. Reports from other European countries on MDMA use have also shown relatively stable trends, following a decrease in the beginning of the century (European Monitoring Centre for Drugs and Drug Addiction, 2020).

Cocaine use increased significantly from 2017 to 2019 by 139% ($p < 0.05$, t-test). These results are in accordance with other European reports on rising use of cocaine (European Monitoring Centre for Drugs and Drug Addiction, 2020; González-Mariño et al., 2020). Availability of cocaine has increased in Europe in recent years with record breaking numbers of seized quantities (European Monitoring Centre for Drugs and Drug Addiction, 2020). This has led to higher purity of the drug in Iceland and lower prices (National Police Commissioner of Iceland, 2019). The consequences can be seen in an increase in cocaine dependencies in rehabilitation centres in Iceland, including a rise in the number of new admissions and number of patients (National Police Commissioner of Iceland, 2019; Tyrfingsson, 2019). A similar trend was

observed before the financial crash in Iceland in 2008 where an increase in cocaine use was detected but decreased again after the crash. The improving economic status in Iceland due to expanding tourism could therefore be a contributing factor in rising use of the drug. In June 2020 during the COVID-19 pandemic, estimated cocaine use by WBE had dropped significantly by 60% ($p < 0.05$, t-test) compared to April 2019. These results indicate a changed consumption pattern of the drug during the pandemic. During the time of the sample collection in June 2020, all nightclubs in Reykjavik had restricted opening hours. This could cause the consumption to take place in residential homes to a larger extent, leading to a change in the choice of substances.

Cannabis use by WBE was relatively stable from 2017 to 2019. Surveys among young people on cannabis use in Europe have either shown stable or increasing trends in recent years, supporting data from Reykjavik (European Monitoring Centre for Drugs and Drug Addiction, 2020). In June 2020 during the COVID-19 pandemic, a 35% increase ($p = 0.0573$, t-test) in cannabis use estimated with WBE was observed. This further indicates a change in the consumption pattern of illicit drugs in Reykjavik during the pandemic with restricted opening hours of nightclubs and increased consumption in residential homes.

Trends between weekdays in illicit drug use can be determined using WBE where the difference between used amounts during weekends (Saturday to Sunday) are compared to other weekdays. Of all the illicit drugs examined, the most extensive increase during weekends compared to weekdays was observed in MDMA use, ranging from 42% to 154%. This increase was significant during all sampling periods ($p < 0.05$, t-test). Increased use of MDMA during weekends has previously been reported indicating its recreational use (Löve et al., 2018; Ort et al., 2014; Thomas et al., 2012). Similar but less extensive trends were observed for cocaine, ranging from 29% to 68% increase during weekends, with the exception of only a 10% increase in June 2020 during COVID-19. This increase was significant during ten out of eleven sampling periods between 2017 and 2020 ($p < 0.05$, t-test). These results suggest that cocaine, like MDMA, is used recreationally during weekends in Iceland corresponding with previous reports (Löve et al., 2018; Ort et al., 2014; Thomas et al., 2012). Trends between weekdays in amphetamine use were minimal compared to MDMA and cocaine with up to a 31% increase during weekends, which was only significant in six out of eleven sampling periods. Trends between weekdays and weekends in methamphetamine use varied considerably ranging from no increase during weekends to a 93% increase. Changes in cannabis use between weekdays

were not significant from 2017 to 2019 but showed a significant increase by 42% ($p < 0.05$, t-test) during weekends in June 2020. This further indicates a shift in the consumption patterns of illicit drugs during the COVID-19 pandemic where recreational use of stimulant drugs such as cocaine consumed in nightclubs has been replaced with cannabis use in residential homes. Nevertheless, it should be taken into account that only one 7-day sample collection was performed during the COVID-19 pandemic and therefore does not represent the total period of the pandemic. Results on illicit drug use in Reykjavik by WBE from February 2017 to June 2020 are shown in Figure 15.

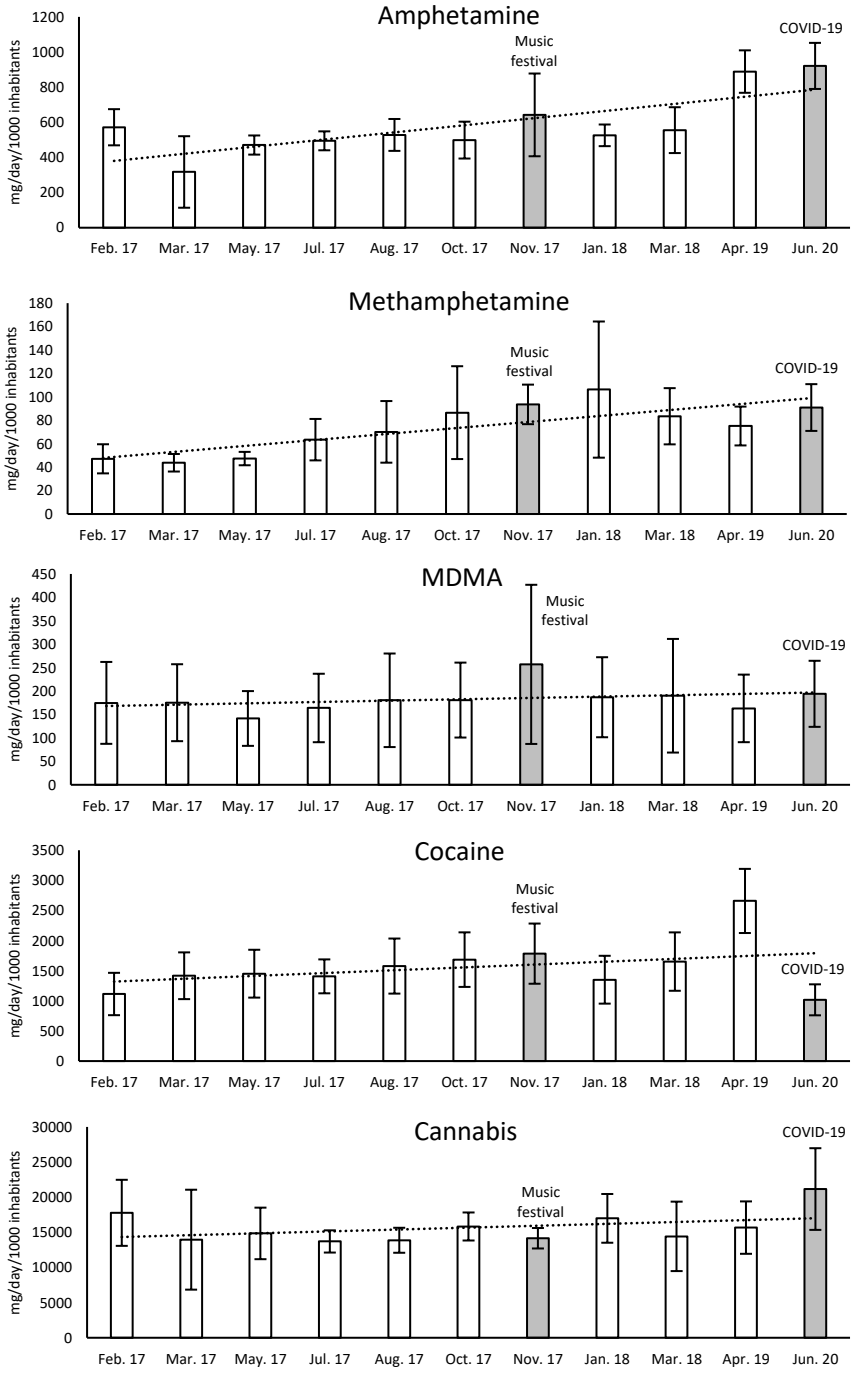

Figure 15. Illicit drug use in Reykjavik by WBE in mg/day/1000 inhabitants during eleven weeklong sampling periods between February 2017 and June 2020

Illicit drug use during special events was examined. Wastewater samples were collected in November 2017 when a yearly music festival (Iceland Airwaves) was held in Reykjavik from Thursday to Sunday. During the music festival an extensive increase in the use of MDMA and cocaine was observed. Signs of an increase in amphetamine use was also detected but to a lesser extent compared to MDMA and cocaine. The most significant increase ($p < 0.05$, t-test) was observed in MDMA use from Saturday to Sunday (192% increase). MDMA use did not begin to rise significantly until the weekend, although the music festival started two days earlier. Unlike MDMA, a significant rise in cocaine use ($p < 0.05$, t-test) was observed during the total period of the music festival from Thursday to Sunday (55% increase). An increase in amphetamine use by 66% was also observed during the music festival. These results further indicate the recreational use of MDMA, cocaine and amphetamine during special events as well as weekends in Reykjavik. Similar results have been reported in other European studies (Krizman-Matasic et al., 2019). Methamphetamine and cannabis use showed no significant trends between weekdays during the music festival. These results are in accordance with previous reports on the stable daily use of Cannabis (Krizman-Matasic et al., 2019; Mackuľak et al., 2019; Thomas et al., 2012). Trends in stimulant drug use during the music festival are shown in Figure 16.

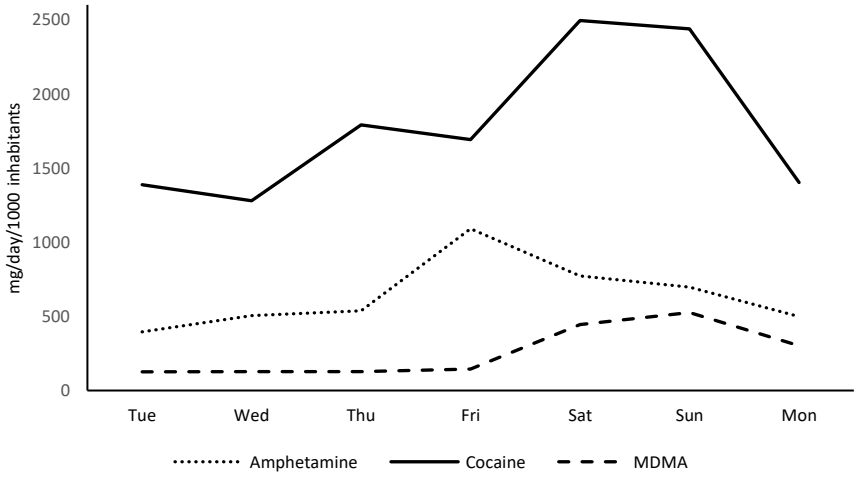

Figure 16. Trends between weekdays in amphetamine, cocaine and MDMA use during a special event held in Reykjavik in November 2017 from Thursday to Sunday.

4.7 Comparison with other indicators of illicit drug use

Results on the comparison of illicit drug use in Reykjavik by WBE with two other indicators of drug use, data on DUI cases and seized amounts of drugs, are presented in Paper III.

The analysis of biological samples from drivers is based mainly on preliminary drug testing performed by the police. The compounds in question are amphetamines together as a package (amphetamine, methamphetamine and MDMA), cocaine and cannabis. The number of positive amphetamine cases increased by 117% from 2014 to 2019 with the largest rise between 2016 and 2019. A decrease by 20% was observed in 2020 during the COVID-19 pandemic. This is somewhat in accordance with data by WBE which also showed signs of increased use between 2018 and 2019. A large increase by 140% in positive methamphetamine cases was observed from 2014 to 2019 but like for amphetamine, a decrease by 31% in 2020. The number of positive methamphetamine cases was nevertheless considerably lower compared to amphetamine. These results are reflected in data obtained by WBE which indicate that amphetamine is more commonly used than methamphetamine. The number of positive DUI cases for MDMA increased by 61% from 2014 to 2019, mostly between 2015 and 2018. The number of MDMA cases dropped slightly from 2018 and 2019 and further by 42% in 2020. These results correspond with data by WBE to a certain extent which indicate stable MDMA use since the beginning of 2017. The extensive drop in the number of MDMA cases during the pandemic was not detected by WBE. The largest rise was observed in DUI cases positive for cocaine with a five-fold increase between 2014 and 2019. This increase was largely between 2016 and 2019. These results are strongly reflected in rising amounts in wastewater from 2017 to 2019, indicating increased use of the drug. Nevertheless, an extensive decrease in the number of DUI cases positive for cocaine was observed in 2020 during the pandemic, which corresponded with data by WBE. Considerable fluctuations were seen in the number of positive cannabis cases with a decrease between 2014 and 2015, an increase by 54% between 2015 and 2017 but relatively stable numbers between 2017 and 2019, which corresponds with data based on wastewater analysis. Taking these fluctuations into account, an upwards trend in positive DUI cases for cannabis was observed from 2015 to 2019. In 2020, a decrease in the number of DUI cases positive for cannabis was observed which did not correspond with data by WBE showing an increase in use. In summary, a decrease in the number of DUI cases in 2020 was observed for all the investigated compounds which did not correspond with data by WBE. A likely cause is the drastic change in

commuting patterns and restrictions on opening hours of nightclubs leading to the consumption of illicit drug to take place in private homes to a larger extent. Trends in positive DUI cases for commonly used illicit drugs from 2014 to 2020 are shown in Figure 17.

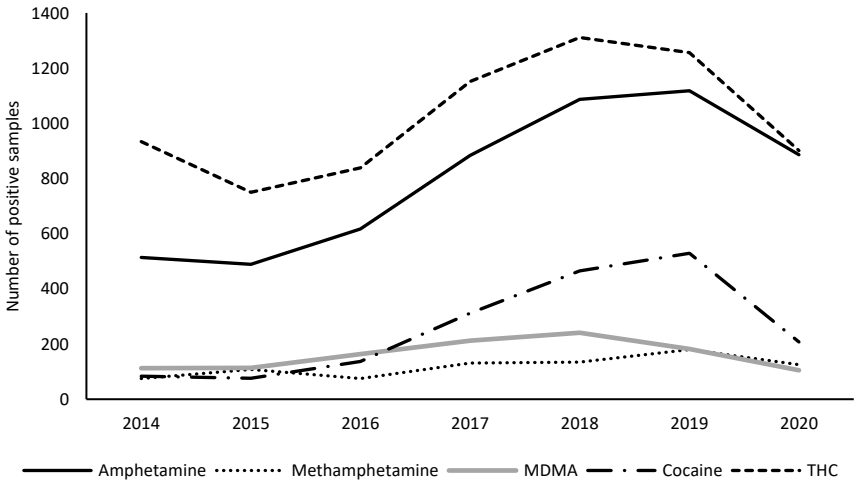

Figure 17. The number of positive DUI cases for commonly used illicit drugs from 2014 to 2020.

Substantial fluctuations were observed in seized amounts of amphetamine in powder form from 2014 to 2019 with no significant trends. However, an indication of increasing import of amphetamine liquid intended to produce amphetamine powder was observed between 2017 and 2019. Reports on the abundance of amphetamine on the market support that the drug is being produced locally to a larger extent (National Police Commissioner of Iceland, 2019). Increased availability of amphetamine on the market due to local production could explain rising levels in wastewater detected in 2019. This rise could be explained by either an increase in the number of users or purity of the drug. From 2006 to 2014, seized amounts of methamphetamine were minuscule but began to increase in 2015 with the largest amounts seized in 2019, indicating limited but increasing use of the drug. Amounts of seized methamphetamine were nevertheless very small compared to amphetamine, supporting data by WBE and numbers of DUI cases. No significant trends were observed in seized amounts of MDMA from 2006 to 2019. Negligible amounts of MDMA were seized from 2016 to 2019. With stable amounts in wastewater from 2017, this suggests that MDMA is being imported into the country without being discovered by the authorities. A 23-fold increase in seized amounts of

cocaine was observed from 2014 to 2019 (Figure 18). This indicates that the use of cocaine in Iceland is on the rise, further supporting data by WBE and the number of positive DUI cases. A steady decrease in seized amounts of cocaine was seen after the financial crash in 2008 until the year 2014 (78% decrease). This decrease could be explained by the declining economic status causing other stimulant drugs such as amphetamine to be preferred due to lower prices. In 2014 the economic status in Iceland began to improve with the effect of high supply and low prices. This caused cocaine use to increase again. Seized amounts of illicit drugs from Iceland are based on large seizures by the police and customs. The purity of drugs on the street is not analysed in Iceland and therefore not known. Comparison of data by WBE and the purity of drugs at street level would give more accurate and detailed information where a distinction between trends in the number of users versus the purity of drugs would be possible.

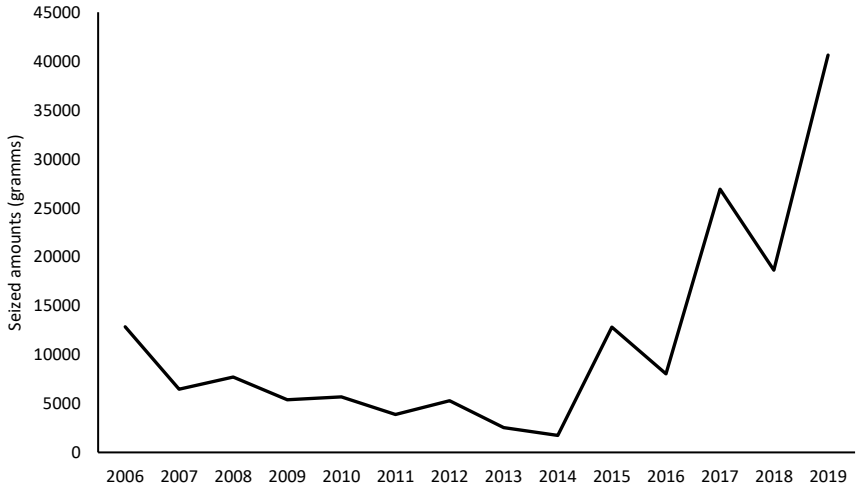

Figure 18. Seized amounts of cocaine in grams from 2006 to 2019.

A change was seen in seized amounts of cannabis after the financial crash with a shift from illegal import of cannabis produce such as hash to locally cultivated cannabis plants. This was observed in the low proportion of seized amounts of cannabis at the national border compared with seizures at local production sites. Average seized amounts of marijuana increased more than 8-fold between 2009 and 2019 compared to 2006 and 2008, but the average seized amounts of hash from 2009 to 2019 decreased by 88% compared to 2006 to 2008. An abundance of locally cultivated cannabis causes the

availability of the drug to remain high as well as the demand. The shift from seized amounts of hash to locally cultivated marijuana in Iceland from 2006 to 2019 is shown in Figure 19.

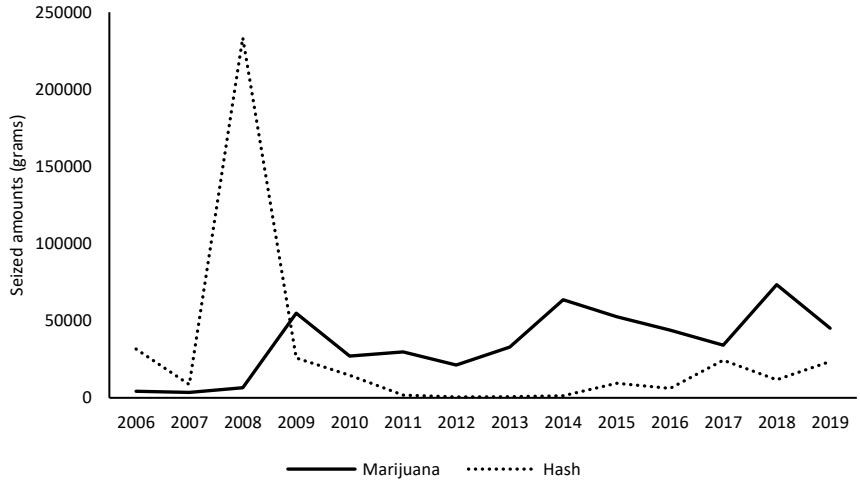

Figure 19. Seized amounts of hash and marijuana by the police from 2006 to 2019.

Good comparability was between data by WBE and other indicators of drug use. Similar results from other European countries have been published with the simultaneous interpretation of multiple indicators of drug use (Kankaanpää et al., 2016). This comparison further allows for a more detailed and comprehensive estimation of drug use in Iceland. A more complete dataset where sample collection is conducted regularly throughout the year would give a more detailed estimation of illicit drug use in Iceland, excluding uncertainties related to temporal variability (Gunnar and Kankaanpää, 2019).

4.8 Analysis of pharmaceuticals in wastewater

Pharmaceuticals with known abuse potential and addictive properties were selected for analysis in wastewater from Reykjavik. Opioids such as codeine, morphine and tramadol are commonly used in Iceland according to the Icelandic Prescription Medicines Register and were therefore chosen for analysis. The ADHD drug methylphenidate was also chosen as research has shown it to be the drug of choice for intravenous users in Iceland (Bjarnadottir et al., 2015). Data from the Prescription Medicines Register containing the

number of DDDs for filled prescriptions per day per 1000 inhabitants between January 2017 and December 2019 was compared with results obtained with wastewater analysis. The number of DDDs/day/1000 inhabitants for the prescription drugs chosen are shown in Figure 20.

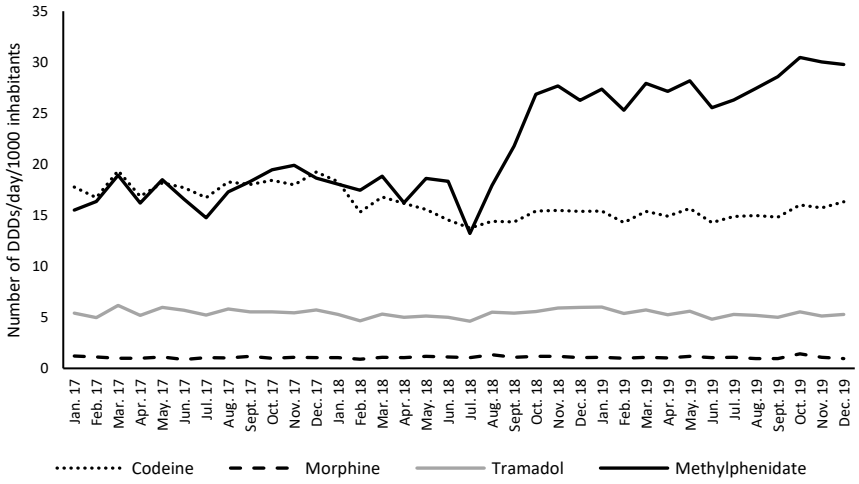

Figure 20. Data from the Prescription Medicines Register on the number of DDDs/day/1000 inhabitants based on filled prescriptions of morphine, codeine, tramadol and methylphenidate from January 2017 to December 2019.

Codeine use by WBE was relatively stable from February 2017 to April 2019 with some fluctuations between sampling weeks. Nevertheless, considerable variability between days was observed in codeine use which was not explained by increased use during the weekends or special events. The number of DDDs for filled prescriptions of codeine showed some fluctuations as data by WBE but was relatively stable overall. Codeine is often prescribed for a short amount of time for mild and moderate pain after e.g. injuries or surgery which could explain fluctuations between days (Moore et al., 1997). The total average amount of codeine in wastewater from February 2017 to April 2019 was 67% higher than the average amount of morphine. This is in accordance with the number of DDDs of filled prescriptions which were 15-fold higher for codeine compared to morphine. Morphine use by WBE remained very stable during the total sampling period from February 2017 to April 2019 and showed extremely low variability between days. These results are in accordance with data obtained from the Icelandic Prescription Medicines Register which showed very stable numbers of DDDs from 2017 to 2019. Although the comparison between data from the Prescription Medicines Register with data by WBE does show similar trends, it is not feasible to

compare the data in parallel as information on filled prescriptions does not necessarily indicate that the drug has been taken at that time point. Filled prescriptions could indeed be used at a later date or not at all. These limitations must be kept in mind when comparing these datasets. Results from the analysis of codeine and morphine in wastewater are shown in Figure 21.

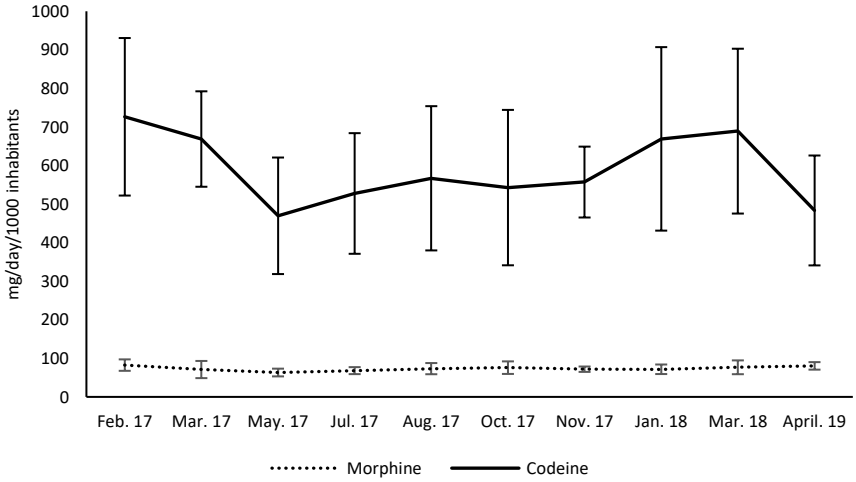

Figure 21. Morphine and codeine use in Reykjavik between February 2017 and April 2019 by WBE in mg/day/1000 inhabitants.

As previously mentioned, there are several uncertainties associated with WBE, including the appropriate selection of correction factors and drug target residues. Previously published studies have reported the analysis of tramadol as opposed to its metabolite O-desmethyltramadol to estimate use of the drug (Bishop et al., 2020; Kim and Oh, 2020; Yargeau et al., 2014; Zhou et al., 2019). Due to the scarcity of published studies, it was decided to analyse both O-desmethyltramadol and tramadol in all wastewater samples from Reykjavik. The results were compared to determine which analyte would be best suited for further analysis. Results for tramadol showed relatively stable use during the total sampling period from February 2017 to April 2019, ranging from 504 mg/day/1000 inhabitants to 791 mg/day/1000 inhabitants. Variability between days was also low with minimal changes between weekdays and weekends. Unlike tramadol, results for O-desmethyltramadol indicated both higher use of tramadol and showed more fluctuations between sampling weeks, ranging from 537 mg/day/1000 inhabitants to 1850 mg/day/1000 inhabitants. Variations between days were also considerably larger compared to tramadol. Data from the Prescription Medicines Register (Figure 20) shows that the number of DDDs for filled prescriptions of tramadol between January

2017 and December 2019 was extremely stable. Assuming that filled prescriptions are consumed thereafter and illegal import of tramadol is excluded, a more stable use of tramadol would be expected rather than fluctuating use. Tramadol and O-desmethyltramadol are excreted in similar amounts in urine (Table 6) and therefore differences in excretion percentages could not explain these inconsistencies. Analytical challenges could be a reason for high variability of O-desmethyltramadol levels in the wastewater. A corresponding internal standard for O-desmethyltramadol was not available and therefore tramadol-d3 was used as an internal standard. This could be a contributing factor to the difference in back-calculated results based on O-desmethyltramadol compared to tramadol. Further method development would be appropriate to determine if this is a causing factor. These results therefore indicate that tramadol is the better option for wastewater analysis supporting previously published studies (Bishop et al., 2020; Kim and Oh, 2020; Yargeau et al., 2014; Zhou et al., 2019). The results from the analysis of tramadol and O-desmethyltramadol in wastewater from Reykjavik are shown in Figure 22.

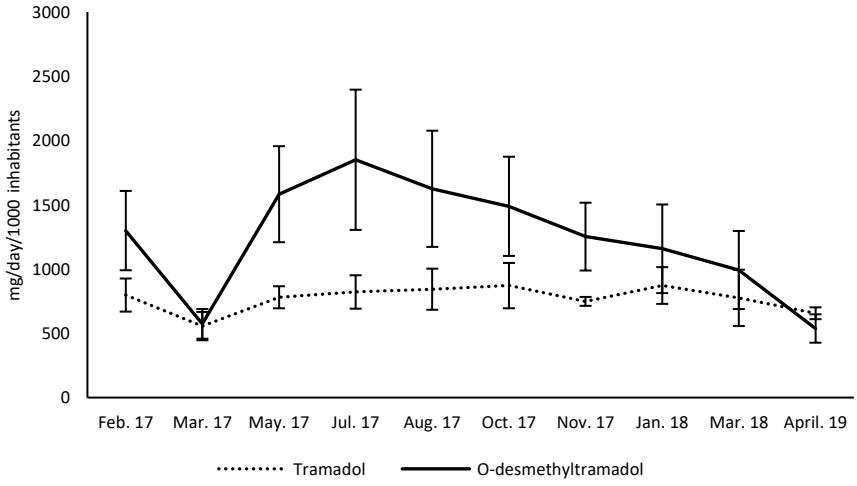

Figure 22. Tramadol use in Reykjavik in mg/day/1000 inhabitants between February 2017 and April 2019 based on the analysis of both tramadol and O-desmethyltramadol in wastewater.

Published data on methylphenidate use estimated with WBE is limited (Bishop et al., 2020; Burgard et al., 2013; Gushgari et al., 2018). The uncertainty related to the choice of an appropriate drug target residue and correction factors is known. Therefore, it was decided to analyse both methylphenidate and its metabolite ritalinic acid to further decide which

compound was more suitable for analysis in wastewater. Results from the analysis of methylphenidate showed considerable fluctuations during the total sampling period from February 2017 to April 2019, ranging from 772 mg/day/1000 inhabitants to 1740 mg/day/1000 inhabitants. Estimated methylphenidate use based on the analysis of ritalinic acid showed more stable results, ranging from 412 mg/day/1000 inhabitants to 830 mg/day/1000 inhabitants. The results also showed that the estimated average use of methylphenidate was higher when the parent compound was analysed in wastewater as opposed to the metabolite. Variability between days was also considerably higher when methylphenidate was analysed compared to ritalinic acid which was not explained by trends between weekdays and weekends. The excretion percentage of ritalinic acid in urine is 60-81% and is much higher than methylphenidate where less than 1% is excreted in urine. The low excretion percentage of methylphenidate increases the risk of uncertainty and could explain the inconsistency between results. It was also observed that in all cases the use dropped during weekends. This is in accordance with a common instruction of use for this pharmaceutical to cease use during weekends. Results show that ritalinic acid would be a more appropriate analyte for the estimation of methylphenidate use as opposed to the parent compound. The reason primarily being that an analyte with a higher excretion percentage is more reliable than when it is very low. And secondly, when comparing levels of both the parent compound and metabolite, the amounts of ritalinic acid are more stable and show less variability between days. When compared with data retrieved from the Prescription Medicines Register, an increase was observed in the number of DDDs of filled prescriptions from July 2018 to October 2018 but showed stable numbers before and after that period (Figure 20). This can be explained by a change in the authorized amounts of dispensed drug in July 2018, which caused the number of filled prescriptions to increase (Védís Helga Eiríksdóttir, Prescription Medicines Register of Iceland, written communication). If the effect of this change in drug dispensing is excluded, used amounts of methylphenidate were stable. Methylphenidate use based on the analysis of ritalinic acid in wastewater is therefore comparable with data obtained from the Prescription Medicines Register indicating stable use of the drug from 2017 to 2019. The results from the analysis of methylphenidate and ritalinic acid in wastewater from Reykjavik are shown in Figure 23.

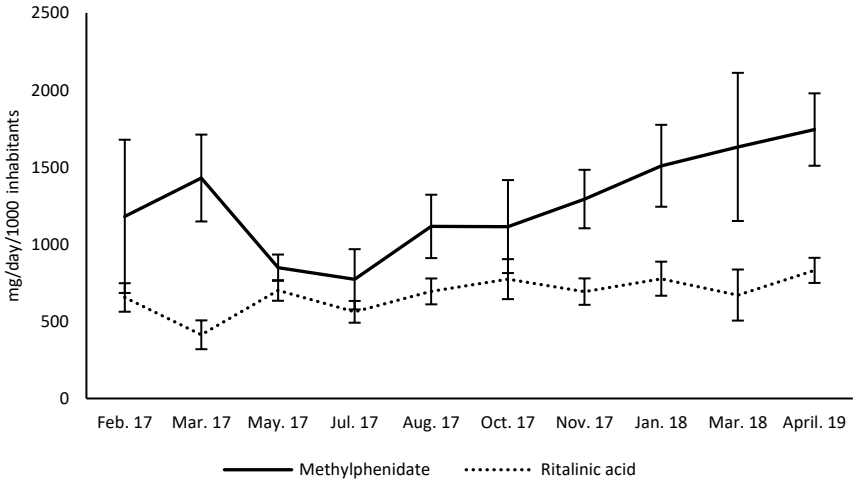

Figure 23. Methylphenidate use in Reykjavik in mg/day/1000 inhabitants between February 2017 and April 2019 based on the analysis of both methylphenidate and ritalinic acid in wastewater.

4.9 Spatial trends in illicit drug use in the Nordic countries

Trends in stimulant drug use in the Nordic countries by WBE are presented in Paper IV. Wastewater samples from five Nordic capitals, Reykjavik, Stockholm, Oslo, Helsinki, and Torshavn, were collected in the spring and summer of 2016. Samples from Reykjavik, Stockholm, Oslo, and Torshavn were analysed using SPE micro-extraction combined with large volume injection and post-loop mixing (Paper II). Results from the analysis of stimulant drugs in the wastewater of five Nordic capitals is shown in Figure 24.

Results showed that amphetamine loads were highest in Reykjavik followed by Stockholm, Oslo, and Helsinki. Amphetamine was not detected in wastewater samples from Torshavn. High variability of results from Reykjavik was observed compared with other cities with a 29% increase during weekends. Samples from Reykjavik were collected in the summer of 2016 but samples from other cities in the spring of 2016. This inconsistency could cause an overestimation of amphetamine results from Reykjavik compared with other cities. Special events are commonly held in the summertime in Reykjavik where the use of illicit drugs such as amphetamine have been known to increase to a larger extent compared to normal weeks. Nevertheless, when temporal trends in amphetamine use are observed in Paper III, minimal differences were observed between amphetamine loads in the spring and

summer of 2017 during normal weeks. Amphetamine loads from Stockholm were also high which was in accordance with reports on seized amounts, showing that amphetamine is the second most commonly seized drug in Sweden after cannabis (European Monitoring Centre for Drugs and Drug Addiction, 2019d). Results did not indicate recreational use of amphetamine in Stockholm and Oslo which is consistent with previous reports (Thomas et al., 2012). Helsinki showed a 33% increase during weekends indicating recreational use of the drug similar to Reykjavik, which agrees with published data (Kankaanpää et al., 2016).

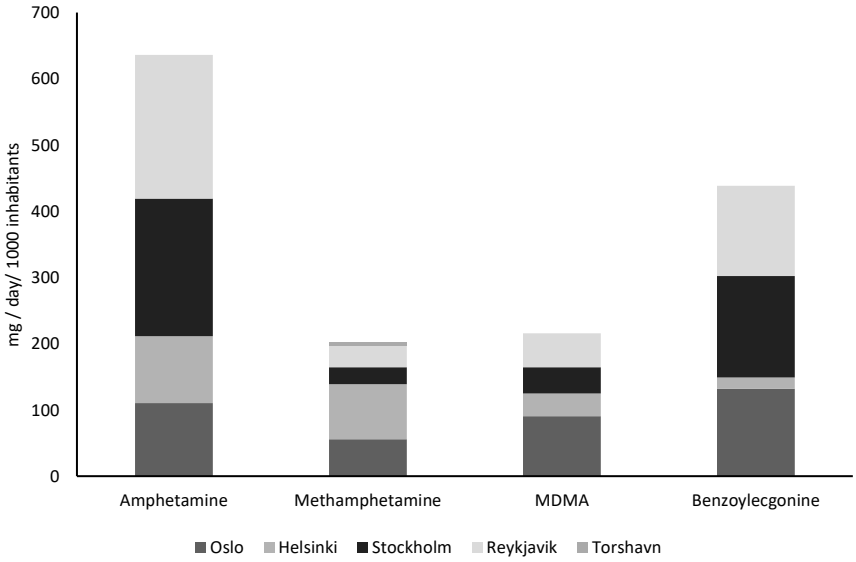

Figure 24. Average loads of stimulant drugs in wastewater from five Nordic capitals in mg/day/1000 inhabitants.

Methamphetamine was detected in wastewater samples from all cities included in the study. Helsinki had the highest methamphetamine loads, followed by Oslo, Reykjavik, Stockholm, and Torshavn. High amounts of methamphetamine were found in wastewater from Helsinki compared with previous published reports (Kankaanpää et al., 2014; SCORE, 2020). This indicated substantial fluctuations in the use of methamphetamine in Helsinki, depending on its availability, purity and price (Gunnar and Kankaanpää, 2019). Methamphetamine loads were also high in wastewater from Oslo. These results corresponded with published findings on high methamphetamine use in Oslo compared to other European cities but also indicated a decrease in use compared to previous years (Bramness et al., 2015; Ort et al., 2014; SCORE,

2020; Thomas et al., 2012). Methamphetamine loads in wastewater were low from both Reykjavik and Stockholm indicating minimal use of the drug compared to amphetamine, supporting previously published data (Östman et al., 2014; SCORE, 2020). Methamphetamine was detected in wastewater from Torshavn, but average loads were extremely low. Only 4% of the population of Torshavn contributed to the wastewater samples leading to a high degree of uncertainty. These results nevertheless indicate that methamphetamine use is limited in Torshavn, but further interpretation was not possible due to the known uncertainties. Some evidence of recreational methamphetamine use in Oslo was observed with a 28% increase during weekends. No significant difference between weekdays and weekends was detected in methamphetamine loads from Helsinki, Stockholm, and Reykjavik.

MDMA was detected in wastewater samples from all cities except for Torshavn. The largest MDMA loads were in wastewater from Oslo, followed by Reykjavik, Stockholm, and Helsinki. MDMA loads in wastewater from Oslo were substantially higher compared to previous reports indicating a rise in MDMA use (SCORE, 2020; Thomas et al., 2012). An increase in MDMA seizures in the years before also support these results (European Monitoring Centre for Drugs and Drug Addiction, 2019c). MDMA loads from Reykjavik were high. These results nevertheless represent a summer week with several special events. This could cause higher levels in wastewater compared to normal weeks due to increased recreational use. MDMA loads from Stockholm and Helsinki were similar. MDMA loads in wastewater from Stockholm was higher than previously reported which corresponds with an extensive increase in seized amounts of the drug in the years before (European Monitoring Centre for Drugs and Drug Addiction, 2019d; Thomas et al., 2012). Previous reports have also shown an increase in MDMA loads from Helsinki as well as an increase in seized amounts (European Monitoring Centre for Drugs and Drug Addiction, 2019b; Kankaanpää et al., 2016, 2014). In all cities, a substantial rise in MDMA loads was observed during weekends, from an 83% increase in Reykjavik to a 186% increase in Stockholm. These trends confirm that MDMA is used recreationally to a great extent as previously reported (Kankaanpää et al., 2016; Thomas et al., 2012).

Cocaine and its metabolite benzoylecgonine were detected in wastewater samples from all cities except Torshavn. The highest amounts of benzoylecgonine, representing cocaine use, were analysed in wastewater from Stockholm, followed by Reykjavik, Oslo, and Helsinki. High loads of benzoylecgonine in wastewater from Stockholm indicated an increase in cocaine use, which corresponded with data on increasing amounts of seized

cocaine (European Monitoring Centre for Drugs and Drug Addiction, 2019d). Benzoylecgonine amounts in wastewater from Reykjavik and Oslo were similar to Stockholm. Corresponding levels of benzoylecgonine in wastewater from Reykjavik had been reported earlier that same year indicating low seasonal variability of the drug. Helsinki had the lowest benzoylecgonine loads of the cities included in this study. Although levels were low in Helsinki, previously published data showed rising levels in wastewater from 2012 to 2015 (Kankaanpää et al., 2016, 2014). Results strongly indicated that cocaine is used recreationally in all the above-mentioned cities, ranging from a 64% increase in Oslo to 94% increase in Stockholm during weekends. However, to a lesser extent compared to MDMA. The recreational use of both MDMA and cocaine with increased use during weekends has been previously described (Thomas et al., 2012).

The metabolite coca-ethylene, produced after the consumption of both cocaine and alcohol, was also analysed in the wastewater of four of the five cities included in this study (Reykjavik, Stockholm, Oslo, and Torshavn). Coca-ethylene was detected in wastewater samples from Reykjavik, Stockholm, and Oslo with comparable results. The results showed an increase in coca-ethylene levels during weekends in wastewater from all cities. This further indicates that the co-consumption of cocaine and alcohol can be associated with recreational use.

5 Summary and conclusions

WBE was proposed to estimate the use of illicit drugs and pharmaceuticals in Iceland to compliment previously used methods. WBE has been proven to provide objective results on the drug use of total populations in near-real time, where both temporal and spatial trends can be observed.

A reliable analytical method was adapted and validated for the simultaneous analysis of commonly used illicit drugs in Iceland (cocaine, amphetamine, methamphetamine, MDMA, and cannabis) and pharmaceuticals with known abuse potential (codeine, morphine, tramadol, and methylphenidate). To reach detectable levels, a sample extraction method was adapted to Icelandic conditions. Known uncertainties were addressed such as loss of compounds due to degradation or absorption which was prevented by avoiding filtration and acidification of samples. Recoveries were acceptable for the chosen analytes and were between 94% and 113% except for O-desmethyltramadol with a recovery of 138%. Analytical performance was assessed with yearly participation in inter-laboratory comparison studies with satisfactory results. A high-throughput SPE micro-extraction method combined with large volume injection and post-loop mixing was also presented for both illicit drugs and pharmaceuticals. By extracting lower volumes of sample compared to other conventional methods, it was possible to reduce the time per sample considerably and costs with lower amounts of internal standards and solvents being used.

Temporal trends in illicit drug use were effectively assessed at eleven time points from 2017 to 2020. Results by WBE showed the largest significant increase for cocaine use from 2017 to 2019 but the use had decreased in June 2020 during the COVID-19 pandemic. Amphetamine use was estimated to be relatively stable from 2017 to 2018 but showed signs of an increase in 2019. Methamphetamine use increased significantly but remained low compared to amphetamine. MDMA use was stable from 2017 to 2020. Cannabis use was stable from 2017 to 2019 but had increased in 2020 during the COVID-19 pandemic. Trends between weekdays showed a significant increase in both MDMA and cocaine use during weekends indicating recreational use of the drugs. The same trends were observed during a music festival held in Reykjavik where MDMA and cocaine use increased significantly. It has therefore been demonstrated that WBE can be effectively used to estimate trends between weekdays and during special events.

Estimation of illicit drug use in Reykjavik by WBE was compared with both seizure data and driving under the influence cases. Results by WBE were in most cases comparable with the other indicators of illicit drugs use. It was therefore established that combining other indicators of illicit drug use with WBE can give a more comprehensive picture of the drug problem in Iceland. Nevertheless, a longitudinal study with regular sample collections would be optimal for more accurate comparisons.

Pharmaceuticals with known abuse potential were analysed in wastewater from Reykjavik. To establish the most appropriate choice of drug target residues, a comparison was made between both methylphenidate and its metabolite ritalinic acid as well as tramadol and its metabolite O-desmethyltramadol. The results showed that measurements of ritalinic acid and tramadol were more stable with less fluctuations between days. Overall, data by WBE showed comparable results with trends based on prescription data.

Spatial trends in illicit drug use were estimated by comparing data by WBE from five Nordic capitals (Oslo, Helsinki, Stockholm, Reykjavik, and Torshavn). The results showed high amphetamine loads in wastewater from Reykjavik and Stockholm compared with the other cities. Methamphetamine was the only illicit drug detected in wastewater samples from Torshavn in very small quantities but showed the highest loads in Helsinki. MDMA loads from Oslo were highest with lower but similar loads from the other cities except Torshavn. Cocaine loads were similar from Oslo, Stockholm, and Reykjavik, but notably lower than Helsinki. Clear variations in MDMA and cocaine loads were observed between weekends and other weekdays indicating that these drugs are popular for recreational use in the Nordic cities.

Both temporal and spatial trends in illicit drug use in Reykjavik have been successfully estimated by using WBE, complimenting other indicators of drug use. For future analysis, it would be optimal to use SPE micro-extraction to minimize both labour intensity and costs. Regular sample collection is also crucial to effectively detect and evaluate emerging trends. Cooperation with local agencies such as health care professionals and the police would be a practical way to utilize results obtained with WBE to encourage preventative measures.

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Original publications

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Paper I

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Improving wastewater-based epidemiology to estimate cannabis use: focus on the initial aspects of the analytical procedure

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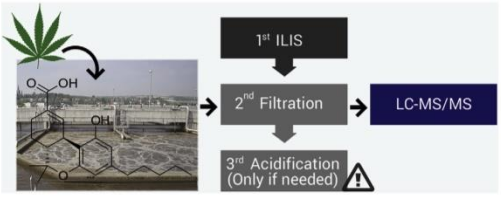
HIGHLIGHTS

- Improvement in the estimation of cannabis consumption through wastewater analysis.
- The order of sample treatment steps is crucial for the determination of THC-COOH.
- Acidification of the wastewater samples should be avoided.
- Results of inter-laboratory exercise support the recommended protocol.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology is a promising and complementary tool for estimating drug use by the general population, based on the quantitative analysis of specific human metabolites of illicit drugs in urban wastewater. Cannabis is the most commonly used illicit drug and of high interest for epidemiologists. However, the inclusion of its main human urinary metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) in wastewater-based epidemiology has presented several challenges and

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concentrations seem to depend heavily on environmental factors, sample preparation and analyses, commonly resulting in an underestimation. The aim of the present study is to investigate, identify and diminish the source of bias when analysing THC-COOH in wastewater. Several experiments were performed to individually assess different aspects of THC-COOH determination in wastewater, such as the number of freeze-thaw cycles, filtration, sorption to different container materials and in-sample stability, and the most suitable order of preparatory steps. Results highlighted the filtration step and adjustment of the sample pH as the most critical parameters to take into account when analysing THC-COOH in wastewater. Furthermore, the order of these initial steps of the analytical procedure is crucial. Findings were translated into a recommended best-practice protocol and an inter-laboratory study was organized with eight laboratories that tested the performance of the proposed procedure. Results were found satisfactory with z-scores ≤ 2.

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1. Introduction

Drug use has not only a negative impact on health and well-being of individuals and people around them, but also represents a clear threat to the stability and security of entire regions and to economic and social development. Cannabis is the most widely cultivated and trafficked illicit drug, responsible for over 75% of drug seizures in Europe [1]. As the most commonly used illicit drug, it is of great interest from an epidemiological point of view. According to the United Nations Office on Drugs and Crime (UNODC), 3.8% of the global population used cannabis in 2014 [2] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimated that 13.3% of young adults (15–34) consumed cannabis in the European Union that same year [3]. Although the use of cannabis has remained stable worldwide over the past years, in some regions, particularly North America and Western and Central Europe, its use has recently increased [2]. The development and use of complementary monitoring tools is important to have a more complete understanding of cannabis use and the impact of new cannabis policies.

Estimating community drug use through the chemical analysis of specific human biomarkers in wastewater has demonstrated its potential to become a useful complementary approach to established drug monitoring tools such as epidemiological surveys, treatment demand and law enforcement data. This technique, referred to as wastewater-based epidemiology (WBE), provides near-real-time information on geographically and temporal drug use patterns, particularly relevant against the backdrop of an ever-shifting drug problem. This quantitative approach is well established to estimate the consumption of cocaine, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) [3–5]. However, in contrast to these substances, the estimation of cannabis using WBE is problematic [3].

The principal active ingredient of cannabis is Δ^9-tetrahydrocannabinol (THC), but in WBE studies the urinary metabolite of THC, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), is used as target biomarker [6]. THC-COOH is specific and, compared to other metabolites, shows high stability over 72 h in wastewater [7,8]. The metabolism of THC is diverse and extensive, a relatively low percentage of THC is excreted as THC-COOH [3,6]. One challenge is therefore the need for more research to better understand the excretion percentage of THC-COOH in order to refine back-calculations to estimate THC consumption. This challenge will not be addressed in the present paper. Another challenge is the analytical determination of THC-COOH in wastewater. Some knowledge gaps associated with physical processes were identified, such as its potential to partition on particulate matter [9,10] and adsorption onto hydroxyl sites present on the surface of glassware [11]. THC-COOH has different physicochemical properties compared to the other conventional illicit drugs (see Tables SI–1). At acidic pH, THC-COOH is present in its non-charged hydrophobic form, which means it may partition to particulate matter, sample containers or filter material, while at neutral pH and the basic pH of natural wastewater the molecule is negatively charged and more hydrophilic. In general, the analytical difficulties and non-instrumental factors have strongly been related to the lower polarity (high lipophilicity) of THC-COOH compared to other illicit drugs when included in multi-residue methods [12–15]. The results of inter-laboratory exercises performed by the Sewage analysis CORe group Europe Network [16] corroborated the difficulties related to the chemical analysis of THC-COOH in wastewater [5]. Although the laboratories involved in those exercises successfully determined THC-COOH in the methanol standards, the recoveries of THC-COOH spiked into wastewater were initially low. This observation suggested that concentrations of THC-COOH in wastewater might be underestimated, probably due to losses during some critical analytical steps.

The present manuscript is a result of studies performed by a working group established within the framework of the pan-European inter-disciplinary network (SCORE), which brings together experts from different disciplines interested in standardizing the WBE approach and in coordinating international studies [17]. The aim of the present work is to investigate and identify the sources of possible bias when analysing THC-COOH in wastewaters and to propose best-practice protocols regarding the initial steps of the analytical procedure. The research is an important step in attempting to provide more accurate estimations of cannabis use through WBE.

2. Materials and methods

This paper describes a study that has been performed by a collaborative group involving 12 institutions, and 10 laboratories. A summary of in-house validated analytical methodologies of each participating laboratory is presented in Table 1 and the full details can be accessed in Tables SI–2 (Supplementary Information file). These multi-residue methods were also applied to measure several illicit drugs in wastewater for WBE monitoring studies organized by SCORE [5].

2.1. Reagents and materials

Analytical standards of THC-COOH and its deuterated analogue were prepared starting from certified ampoules, purchased either from Lipomed AG (Arlesheim, Switzerland) or Cerilliant (Round Rock, TX, USA). All laboratories used THC-COOH-d_3 as isotope-labelled internal standard (ILIS), except Lab 9 who used THC-COOH-d_9.

Table 1
Overview of in-house methods performed by participating laboratories.

Lab#	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9 [a]	Lab 10 [a]
Sample volume	50 mL	5 mL	100 mL of "sample" (25 mL sample + 75 mL ultrapurewater)	50 mL of supernatant	100 mL	125 mL	100 mL	100 mL	n.a.	n.a.
Particulate removal	Filtration 1.6 μm glass fiber filter	Filtration 0.2 μm RC syringe filter	Dilution	Centrifugation	Filtration (1) Whatman No. 41 filter paper (2) 0.2 μm PTFE syringe filter	Filtration 2.7 μm Whatman, glass fiber filter	Filtration (1) 1.6 μm glass microfiber filter GF/A (2) 0.45 μm mixed cellulose acetate & cellulose nitrate	Filtration (1) 1 μm glass fiber filter A/E (2) 0.2 μm PES membrane filter	Filtration 0.2 μm Whatman PTFE syringe filter Primo 1 mL syringe	Filtration (1) 1.6 μm glass microfiber filter GF/A (2) 0.45 μm mixed cellulose acetate & cellulose nitrate filter
pH at extraction	Natural	Natural	Natural	Natural	Natural	Acid	Acid	Natural	n.a.	n.a.
SPE material	Oasis HLB	Strata-XC	Oasis HLB	Oasis HLB	Oasis HLB	Oasis MCX	Oasis MCX	Oasis HLB	n.a.	n.a.
Analytical instrument [b]	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-QqQ	LC-LTQ-FT-Orbitrap	LC-QqQ	LC-QTOF MS
Ionization mode (ESI)	−	−	+	+	+	−	−	+	+	−
Reference	[19]	Unpublished	[20]	Adaptation from Ref. [20]	Unpublished	[10]	Adaptation from Ref. [21]	[22]	Adaptation from Ref. [23]	Unpublished
Instrumental variability [c] (Intra-day, RSD (%))	6% (n = 6)	2% (n = 6)	7% (n = 6)	3% (n = 6)	1% (n = 5)	5% (n = 6)	4% (n = 6)	2% (n = 6)	10% (n = 5)	8% (n = 6)
Instrumental variability [c] (Inter-day, RSD (%))	11% (n = 6)	3% (n = 6)	7% (n = 6)	3% (n = 6)	2% (n = 5)	7% (n = 6)	5% (n = 6)	4% (n = 3)	6% (n = 3)	7% (n = 6)

n.a. not applicable.
[a] Labs 9 and 10 did participate in the interlaboratory study but provided results in preliminary experiments.
[b] QqQ: triple quadrue; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight.
[c] Instrumental variaity was performed using a standard solution of 50 ng/L in solvent.

A range of different filter materials with pore sizes ranging from 0.2 to 2.7 μm were tested: glass fibre, regenerated cellulose, mixed cellulose acetate and cellulose nitrate, and polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF) and polyethersulfone (PES) membranes. Filters were supplied by Pall Corporation (Port Washington, NY, USA), Nalgene (Rochester, NY, USA), Phenomenex (Torrance, USA), Whatman (Dassel, Germany), Millipore (Bedford, MA, USA), VWR International (Radnor, PA, USA) and Agilent (California, USA).

The solid-phase extraction (SPE) cartridges used for sample concentration and clean-up were polymer-based: cation exchange mixed mode (Oasis MCX or Strata-XC), or neutral hydrophilic-lipophilic balanced (Oasis HLB). Amino silica-based Strata NH_2 cartridges were used for additional extract clean up by Lab 6. Oasis and Strata cartridges were supplied by Waters (Milford, MA, USA) and Phenomenex (Torrance, USA), respectively (see Tables SI–2).

During preliminary tests vials of different materials were tested: glass and polypropylene (PP).

2.2. Analytical methodology

Instrumental analysis was performed with liquid chromatography coupled to mass spectrometry (LC-MS). In all cases, chromatographic separation was performed using reversed-phase LC columns. Eight laboratories used low resolution MS and two used high resolution MS. Electrospray ionization (ESI) was used in all cases, in either positive or negative mode. More information regarding instrumental parameters can be found in Tables SI–2. Statistical analysis of results was performed with GraphPad Prism version 5.01.

2.3. Experimental

Preliminary experiments were set up in order to identify possible sources of bias regarding the sample preservation and treatment. In all experiments, two types of matrices were included: ultrapure water and filtered wastewater (free of solid particles). Samples were spiked at a sufficiently high concentration level (50 ng mL^{-1}) in order to perform analysis without further pre-treatment. The sample pH reduction was recommended as one of the WBE "best practice" requirements [18] to decrease the bacterial degradation and increase the sample stability. However, a study performed by Senta and colleagues [8] indicated enhanced pre-analytical losses of THC-COOH when samples were filtered at pH 2. Therefore, we included pH adjustment as a parameter in our experiments. These preliminary experiments were performed by multiple laboratories in the consortium. Results were evaluated with the recovery, expressed as percentage (%), and defined as the relative response of THC-COOH divided by the deuterated response and compared to t = 0. In addition, laboratories were asked to evaluate their instrumental variability (expressed as relative standard deviation, RSD%) by analysing at least 5 replicates over 3 days.

2.3.1. Freeze-thaw cycles

The effect of multiple cycles of freezing and thawing of samples containing THC-COOH was evaluated by spiking 20 mL of matrix at 50 ng mL^{-1} THC-COOH and distributing aliquots of 0.5 mL in 2 mL glass vials. Each vial was exposed to a different number of freeze-

thaw cycles: 0, 1, 2, 5, 10 and 20 (n = 3 in every case). After all freeze-thaw cycles had been performed, the ILIS was added and the vials were analysed by direct injection into the LC-MS. Three laboratories provided results.

2.3.2. In-sample stability

The in-sample stability of THC-COOH was tested at three temperatures (20 °C, 4 °C and −20 °C) over a period of 7 days, with sampling points at 0, 1, 4, 7 days. The matrix (3 mL) was spiked at 50 ng mL^{-1} of the analyte, homogenized and distributed in 3 vials of 2 mL, and each stored at one of the three temperatures. After the experiments, the ILIS was added to each vial and samples were directly injected into the LC-MS system. Four laboratories provided results.

2.3.3. Filtration

The effect of sample filtration prior to analysis was assessed at natural pH (~7.5) and acidic pH (samples adjusted to pH 2.5). From 20 mL of THC-COOH spiked matrix at 50 ng mL^{-1} level, 1 mL was transferred into a glass vial for direct analysis while the rest was filtered. Different types of filters were used: (1) type GF/A glass microfiber filters + cellulose nitrate and acetate filters, (2) type A/E glass fibre filters + PES membrane filters, (3) type GF/C glass fibre filters, (4) regenerated cellulose filters + PES membrane filters. The filtered aliquots were spiked with ILIS and directly injected into the LC-MS system. The resulting recovery was compared to the non-filtered sample, and the loss during filtration was calculated as follows:

$$1 - (\textit{average recovery filtered} / \textit{average recovery nonfiltered})$$

Four laboratories provided results.

2.3.4. Sorption

The potential sorption of THC-COOH to the different container surfaces was investigated by storing 1 mL of matrix spiked with THC-COOH at 50 ng mL^{-1} level in vials of two different materials: glass and polypropylene (PP) (n = 3). The sample pH was considered as a second variable. Therefore, two pHs were investigated: natural pH (7.5) and acidic pH (pH adjusted to 2.5). An aliquot was taken after a determined number of days (storage at 4 °C: 0, 1, 4 and 7 days), spiked with the ILIS and directly analysed by LC-MS. Three laboratories provided results.

2.3.5. Order of preparatory steps

In addition to the preliminary experiments described above, the order of sample preparation steps, often performed prior to SPE, was evaluated. The steps were: ILIS addition, sample filtration and pH adjustment (acidification). To do so, one wastewater sample spiked at 800 ng L^{-1} was divided into 4 sub-samples. The order of steps for each of the sub-samples was varied. Samples were subsequently extracted and analysed using the validated methodology of the one laboratory (Lab 6) that performed the experiment.

2.3.6. Inter-laboratory study

From the preliminary experiments, a best-practice protocol was derived stating recommendations on the pre-analytical aspects of the analysis of THC-COOH in wastewater (see below). In order to test the performance of this protocol, an inter-laboratory study was organized with eight laboratories.

40 L of wastewater collected at the entrance of the WWTP in Utrecht (The Netherlands) were used as matrix. A stainless steel mixing tank was used to homogenize the bulk by stirring for 30 min at 400 rpm. Homogenized wastewater was distributed in four 5 L glass volumetric flasks. Wastewater test samples were prepared by KWR as followed: Sample 1, non-spiked, at natural pH (7.5); Sample 2, spiked at low level (72 ng L^{-1}), natural pH (7.5); Sample 3, spiked at high level (720 ng L^{-1}), natural pH (7.5); and Sample 4, acidified to pH 2.5 and spiked at high level (720 ng L^{-1}). The low level (72 ng L^{-1}) and high level (720 ng L^{-1}) were prepared by spiking 0.5 mL and 5 mL of a THC-COOH solution of 0.72 mg L^{-1} (in methanol), respectively into the 5 L bottles and filling up with homogenized wastewater. Each of the prepared samples was distributed in 0.5 L PP bottles. Each bottle contained approx. 450 mL of sample. Bottles were stored in a freezer (−25 °C) overnight in order to be shipped frozen the following day to the participants.

3. Results and discussion

Based on previous inter-laboratory exercises performed by the SCORE consortium [16], the study started from the premise that the instrumental procedures and multi-residue methods of the different laboratories are successful in determining THC-COOH in standard solutions in methanol in the ng mL^{-1} range [17]. Participating laboratories measured THC-COOH in negative- or positive-ESI mode and sample preparation consisted of filtration/dilution/centrifugation and off-line SPE using different types of filters and cartridges (Table 1). Multi-residue methods applied by 3 out of the 8 laboratories consisted in the use of cation exchange mixed mode cartridges for SPE. Although this type of sorbent is most selective towards basic compounds, THC-COOH showed acceptable recovery when interacting with the MCX sorbent through the reversed-phase mechanism [10,21]. ILIS was used as surrogate in order to ensure the analytical quality of the results. Instrumental variability within the participating labs was <10% in all cases (Table 1).

3.1. Effect of sample pre-treatment operations

3.1.1. Freeze-thaw cycles

After 20 freeze-thaw cycles, the THC-COOH concentration showed a slight decrease (≤10%, RSD = 13%) from the initial concentration (see Figure SI-1.1 for wastewater matrix and SI-1.2 for ultrapure water). However, the variability of the result fell within the level of accepted uncertainty of replicate analyses [18] and, therefore, the decrease was considered not significant.

3.1.2. In-sample stability

The in-sample stability results were calculated relative to day 0 (as the mean recovery of each lab before freezing the sample for the first time) (Figure SI-2.1 for wastewater matrix and SI-2.2 for ultrapure water). THC-COOH remained stable in wastewater up to 7 days at all temperatures tested, with relative recoveries between 80 and 120%.

These results confirm the findings reported by González-Mariño et al., 2012 [21] and Heuett et al., 2015 [24] who reported high stabilities up to 3 and 4 months, respectively when stored at −20 °C. González-Mariño also reported losses of THC-COOH when stored at 4 °C, whereas in our study no significant loss was observed at that temperature. In another study [8] that included pH as a second variable, a lower stability of THC-COOH was observed in the acidified samples (54% decrease from the original concentration at pH 2) than in the non-acidified samples (10% decrease from the original concentration at pH 7.4) when stored at 4 °C. This result can be explained by the enhanced adsorption of THC-COOH to solid particulate matter observed at pH 2 as compared to natural pH [9].

3.1.3. Filtration

Details on the individual performance of each filter or filter combination at pH 7.5 and pH 2.5 can be accessed in SI (Tables SI−3). Results presented in Tables SI−3 clearly demonstrate

that filtration has a great impact on the THC-COOH recovery, and that it is highly pH dependent. At acidic pH, THC-COOH is not charged and its lipophilicity increases (logD: 5.1 at pH 2.5 vs 2.4 at pH 7; chemicalize.com). In the case of wastewater at natural pH, the small-volume syringe filter of regenerated cellulose (RC) performed the best (no loss during filtration). However, when filtering larger volumes, the loss amounted to 27–30% independent of the filter material. In the case of acidified wastewater, results invariably showed losses during filtration >75%, which is in a good agreement with findings reported by Senta et al., 2014 [8]. As can be seen in Fig. 1, the average loss during filtration when sample pH was not adjusted (pH ≈ 7.5) amounted to 20% (RSD = 3%). This impact was even higher when wastewater was acidified to pH 2.5 and the loss amounted to 90% (RSD = 1%). Means differed significantly (paired *t*-test, p-value = 7e-4).

3.1.4. Sorption

Results from the sorption experiments are shown in Figure SI-3 (.1 for wastewater matrix and .2 for ultrapure water). Sample pH appears to be a more important parameter than the type of sample container (glass or PP) used. Losses due to sorption to container walls occur more rapidly and to a higher extent at pH = 2.5, as the compound is in its non-charged hydrophobic form.

Altogether, the results from filtration, in-sample stability and sorption tests have identified pH as the variable having the most significant impact on the recovery of THC-COOH. This corroborated that, given the specific physico-chemical properties of THC-COOH, its behaviour is highly dependent on wastewater pH.

3.1.5. Order of preparatory steps

The order of sample preparation steps was evaluated by comparing the recovery obtained in each case. These preparatory steps are performed prior to SPE and employed to prevent the SPE material from clogging [22] or to prevent and correct for in-sample degradation effects as well as matrix effects (i.e. ILIS addition). They are frequently applied when a multi-residue analysis is foreseen [8]. The results for these experiments were in agreement with those assessed in the previous sections.

The conclusion is that sample acidification, if required by the selected enrichment protocols, should be performed only after the sample filtration. Ideally, ILIS should be added before filtration to correct for any potential loss. The results of the preliminary experiments highlighted the influence of pH and the importance of the correct execution order of sample preparation steps before SPE, with sample acidification being critical. When consulting the SCORE inter-laboratory exercise participant laboratories [17], only 5% had performed their analysis using the order of steps identified as the optimal one in this study: 1st ILIS addition 2nd filtration 3rd pH adjustment (only if needed). Therefore, it was decided to perform an inter-laboratory study within the group in order to confirm this hypothesis before making any recommendation.

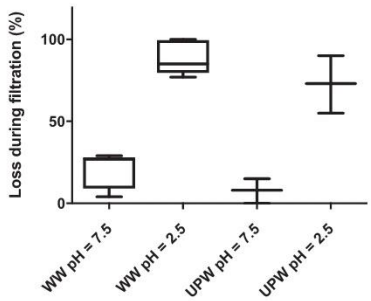

Fig. 1. Losses of THC-COOH during filtration and influence of matrix (WW = wastewater, UPW = ultrapure water) and different sample pH. The data are presented as box plots of grouped results (WW = 4 laboratories, 5 different filter types tested, 3 replicates each; UPW = 3 laboratories, 3 different filter types tested, 3 replicates each) and expressed as percentage of the average recovery of the filtered versus the non-filtered sample. Boxes represent the mean, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values.

3.2. Inter-laboratory study

An inter-laboratory study was performed using the optimal approach identified in the preliminary experiments described above. Four samples were prepared as described in section 2.3.6 and shipped frozen to each participant. All samples were received within 24 h in frozen conditions. Each laboratory was asked to analyse three independent replicates and report THC-COOH concentrations in ng L^{-1} for each sample. The resulting data was tested for homogeneity, the presence of outliers and normality distribution, and z-scores were calculated in order to measure the performance of each laboratory with regard to the group average.

First, the homogeneity of the variances was tested to confirm the correct data comparison (Cochran test). Results showed that the variance for samples 1, 2 and 4 for laboratory 8 was too high (C = 0.738 (sample 1), 0.696 (sample 2), 0.830 (sample 4) > 0.561), therefore those data were removed from the following evaluation. The remaining data set was evaluated for outliers (Grubbs, α = 0.05) and the Shapiro-Wilk normality test (α = 0.05) was applied to determine if the results derived from a normal distribution. All samples passed with following p-values: sample 1, 0.22 (n = 7); sample 2, 0.26 (n = 7); sample 3, 0.34 (n = 8); sample 4, 0.29 (n = 6).

The group's mean average concentration and relative standard deviation per sample was calculated (see Table 2), following the ISO guidelines [25]. For more details, Tables SI–4 shows the mean concentration and standard deviation per laboratory and per sample. Results showed good repeatability (<10%) within laboratories, and reproducibility (≈30%, calculated as the RSD for the mean dispersion), except for sample 4. The reproducibility for samples 1 to 3 is comparable to other inter-laboratory tests [26]. In contrast, the reproducibility for sample 4 was much worse (50%, initially 110% due to the outlier), due to the issues described in previous sections.

Z-scores were calculated to help in the identification of random or systematic errors. To do so, the difference between each individual lab's mean (m) and the group's mean (M) was subtracted, and then divided by the group's standard deviation. This computation provides a value that can be either positive or negative (when the mean is above or below the group's average, respectively), as a measure of the accuracy of each laboratory. The accepted cut-off value is z-score $\leq |3|$, whilst a value between 2 and 3 is considered questionable, in accordance with the IUPAC [27] terminology. Graphical results are presented in Fig. 2.

Z-scores were in general consistently positive or negative for each of the laboratories, which might indicate some type of systematic bias, but within the acceptance criteria. Certain laboratories seemed to be grouped systematically in the lower or higher end, however these groupings appear to be independent of extraction and analysis procedures. Laboratory 8 showed high results for all samples, particularly for samples 1, 2 and 4, as commented above. However, an unambiguous explanation could not be found for this performance.

Table 2
Group's mean (M) per sample expressed in ng L^{-1}, Recovery (R) expressed in absolute value (ng L^{-1}) and percentage (%), and group's relative standard deviation (RSD%) in the inter-laboratory study.

	M	R	RSD (%)	n
Sample 1 – WW blank	814 [b]	–	28% [b]	7 [b]
Sample 2 – WW blank + 72 ng L^{-1}	860 [b]	46 (64%)	27% [b]	7 [b]
Sample 3 – WW blank + 720 ng L^{-1}	1527	807 (112%)	34%	8
Sample 4 [a] – WW blank acidified + 720 ng L^{-1}	442 [b]	−372 (−52%)	50% [b]	6 [b]

R = sample x (x = 2,3,4) – sample 1 (WW blank).
[a] Modified order of anlytical steps, the sample was acidified at KWR before being shipped frozen to the laboratories.
[b] After removal of labratory 8 data.

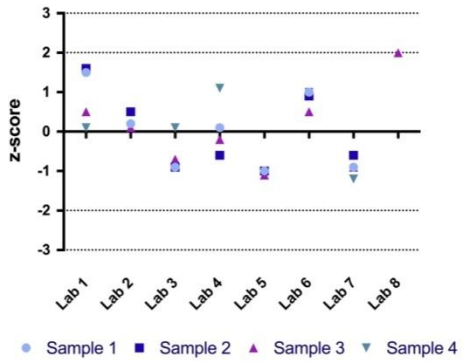

Fig. 2. Inter-laboratory study z-scores per laboratory and sample, calculated as the difference between each individual lab's mean (m) and the group's mean (M) divided by the group's standard deviation.

Recoveries of THC-COOH, defined as the difference between the group's mean for the spiked samples subtracted by the blank sample (see Table 2), were satisfactory (64–112%), with good accuracy from the participating labs for samples 2 and 3, confirming the correct use of the recommended protocol. The mean recovery (52%) observed for the acidified sample 4 demonstrated the negative influence that acidification of the sample may have on recovery.

4. Conclusions

The estimation of cannabis use through wastewater analysis is of high interest. Previous studies have identified several important knowledge gaps as well as analytical challenges. This means that previously published results should be considered with care, as results could have been underestimated.

The results obtained in the current study can be used to define the way forward towards more accurate determination of THC-COOH in wastewater. The adjustment of pH has been identified as a critical step in sample processing. If necessary, samples should be acidified after filtration and only after the ILIS have been added to correct for possible losses. Although the results among all labs varied by approximately 30% and therefore higher than optimal, the proposed protocol was successfully tested, and can, therefore, be recommended for future WBE applications.

Studies regarding THC-COOH sorption to biofilms and solid particles during in-sewer transport would be needed (i) to further reduce uncertainties, as they have already been done for other illicit substances [7,8,23,28,29], as well as (ii) to better understand the cannabis excretion profile in order to achieve a more accurate back-calculation of its consumption.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.08.011.

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Paper II

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A high-throughput solid-phase microextraction and post-loop mixing large volume injection method for water samples

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ABSTRACT

This article presents a novel approach for the analysis of 13 drugs in wastewater for use in wastewater-based epidemiology (WBE) studies. Sample preparation remains one of the principal bottlenecks in modern high-throughput analysis by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The proposed methodology is based on the micro-extraction of small volumes (1 ml) of wastewater using a HLB 96-well microplate and both large volume injection (LVI) and post-loop mixing injection (PLM). With this configuration, the limits of quantification (LOQ) were below the reported environmental concentrations of the target compounds in wastewater. Furthermore, both the complexity of collecting, transporting and storing the wastewater sample, sample preparation time, cost and amount of solvent used are all diminished, enhancing the suitability of this methodology for future WBE studies. A new workflow is also proposed in order to create a virtual specimen library bank for WBE by using high-resolution mass spectrometry (HRMS). The method was validated and the limits of quantification were between 0.2 and 6.3 ng L^{-1}. The relative standard deviations (RSD) for a standard mixture at 200 ng L^{-1} (n = 6) was between 3.4 and 14.4% while the recoveries for the 13 drug target residues (DTR) were between 92 and 110%. The developed and validated method was finally successfully applied to 10 wastewater samples collected from Oslo, Norway.

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1. Introduction

Wastewater-based epidemiology (WBE) has been established as a complementary tool to estimate drug use at the population level by the quantitative measurement of endogenous and exogenous biomarkers excreted by humans in wastewater [1]. Recently WBE has also been shown to be an effective approach for estimating population level human exposure to a wide range of pollutants [2,3]. WBE has the potential to provide real-time data on geographical and temporal trends in illicit drug use [4]. Traditional methods used for this purpose are usually based on population surveys, sales data, clinical cases, seizures or mortality rates related to use, but these approaches lack representativeness, are time consuming and expensive [5].

The WBE procedure consists of several steps involving sample collection, chemical analysis and the drug target residue (DTR) back-calculation, which are subject to a certain number of sources of uncertainty that have been described and progressively diminished by using a harmonized approach [6]. The appropriate collection of representative composite wastewater samples to compensate for the flow fluctuations during the sampling has been described by Ort and colleagues [7], presenting an acceptable uncertainty when estimating the population weighted loads of around 5–10% [6]. Furthermore, wastewater data has been shown to present low temporal representativeness when assessing annual averages [8]. Consequently, the annual estimates for a certain substance based on WBE studies must consist of several stratified random samples (typically 56 samples per year for an acceptable level of sample size related uncertainty < 10% [9]) rather than only one consecutive week as most of the WBE studies, such as the European-wide monitoring for the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [10]. However, increasing the sampling frequency to decrease the annual estimate uncer-

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tainty may therefore imply a greater activity from the wastewater treatment plant (WWTP) operators in order to collect the samples to be analyzed. Therefore, there is a need to develop more suitable and cost-effective alternatives to classic methods for the long-term monitoring of exposure and substance use at community level through WBE [8].

Sample analysis is critical to achieve reliable concentration of the DTR. The uncertainty related to the analytical variability is estimated to be up to 26% [6]. Most of the DTR are found in wastewater in the ng L^{-1} range and therefore a pre-concentration step is usually required [11]. Solid phase extraction (SPE) is the most common procedure for this purpose and large volumes of wastewater are necessary in order to reach the required limits of detection for determining environmental concentrations (between 50 and 1000 mL) [12]. However, the majority of the above procedures are tedious and time-consuming. Miniaturization of the sample preparation has become an alternative in modern high-throughput methods. Solid phase microextraction (SPME) differs from SPE in the ratio sorbent *versus* sample volume. Therefore, all the different SPME configurations are an equilibrium extraction technique since only a small portion of the analyte is extracted from the sample whereas SPE techniques are based on the complete extraction of all the analytes from the sample. Micro-SPE (μSPE) is a miniaturized version of SPE with the same concept of extracting all the analytes but in this case, with a smaller sample volume and a reduced amount of packed sorbent [13].

Large volume injection (LVI) methods are another alternative that provide the advantage of reducing sample preparation steps, improving the reproducibility and minimizing potential contamination of the sample. Furthermore, LVI increases sample throughput at minimal cost [14] and the water sample can be injected in the initial aqueous mobile phase without causing serious peak broadening. However, to date, LVI methods have normally presented low sensitivity with respect to the environmental levels [15], and require modern and very sensitive instruments that are not always available in the analytical laboratories [16].

Ultra-high-performance liquid chromatography (UHPLC) has recently emerged providing higher sensitivity, better separations and improved throughput [5]. UHPLC columns are packed with much smaller particles and support greater pressures that increases the efficiency and decreases the run time. However, UHPLC columns become a problem when using LVI due to lower sample capacity leading to chromatographic distortions such as peak broadening or volume over-load issues [17]. The post-loop mixing (PLM) approach efficiently avoids the above problems by completely diluting the sample into organic mobile phase before the sample reaches the mixer and is diluted and carried to the column by the aqueous mobile phase. The initial elution solvent rate is such that the sample is retained at the head of the column in a narrow band (i.e. A:water 97%; B:methanol 3%). In this case, rather than injecting the wastewater sample directly, the sample is extracted by μSPE and then a larger volume of the eluent is injected into the system directly in organic solvent without reconstitution in water.

At present the main development focus within the WBE field is based on the development of analytical methods for new markers [18–20] and reduction of the uncertainty related to both the in-sewer transformation [21] and the estimation of the population of the WWTP catchment areas [22]. However, due to the relatively low uncertainty and the inter-laboratory exercises for external quality control assurance, the analytical methods have remained unaltered, tedious and inefficient. Therefore, the combination of μSPE with PLM together with LVI provides a perfect compromise between sample throughput, cost, sensitivity and chromatographic separation.

The aim of this study was to develop, validate and apply a novel high-throughput WBE procedure for the analysis of 13 DTR by off-line μSPE-PLM-LVI-UHPLC coupled to tandem mass spectrometry (MS/MS). The selected compounds were amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), benzoylecgonine, cocaine, cocaethylene, atenolol, citalopram, carbamazepine, fexofenadine, methylphenidate, metoprolol and lidocaine. Thus, this procedure will potentially improve the technical and environmental WBE feasibility by: (i) reducing sample preparation and analysis time; (ii) reducing costs; (iii) reducing the amount of solvents needed; (iv) improving the whole method efficiency, (v) making the sample collection and storage easier for the WWTP operator (from 1L to 5 mL or from one big bottle to one small glass vial) and (vi) enabling the creation of a virtual specimen library bank for WBE by archiving and retrospectively analyzing the data acquired in HRMS mode. Finally, to demonstrate the feasibility of this approach, μSPE-PLM-LVI-UHPLC-MS/MS was applied to the analysis of 10 wastewater samples.

2. Experimental

2.1. Reagents and materials

Reference standards for 13 drugs and/or their main metabolites chosen for the analysis were the following: amphetamine, methamphetamine, MDMA, cocaine, benzoylecgonine, cocaethylene, atenolol, citalopram, carbamazepine, fexofenadine, methylphenidate, metoprolol, and lidocaine dissolved in methanol (MeOH) or acetonitrile (ACN) at concentrations of 1 mg mL^{-1} or 100 μg mL^{-1}. Standard solutions of each compound were made in methanol at 100 μg mL^{-1} and then diluted into final mix solutions to a concentration of 10 and 1 ng mL^{-1}. Corresponding isotope-labeled internal standards (ILIS) were amphetamine-d8, methamphetamine-d11, MDMA-d5, cocaine-d3, benzoylecgonine-d3, cocaethylene-d3, atenolol-d7, fexofenadine-d6, metoprolol-d7 and lidocaine-d6 dissolved in MeOH or ACN at concentrations of 100 μg mL^{-1}. The ILIS solutions were made in methanol at 10 μg mL^{-1} and then diluted to a mix working solution at 10 ng mL^{-1}. All reference standards and ILIS were purchased from Cerilliant (Round Rock, TX, USA). The standards and working solutions were stored at −20 °C.

HPLC-grade MeOH was purchased from Rathburn Chemicals Ltd. (Walkerburn, SCT, UK). HPLC-grade ACN was acquired from VWR Chemicals (Oslo, Norway). Ammonium hydroxide (NH_4OH) solution ≥ 25% in water was obtained from Fluka – Sigma-Aldrich (Oslo, Norway) and formic acid (FA) 98–100% (for analysis) was purchased from Merck – Millipore (Oslo, Norway).

2.2. Wastewater samples

Influent wastewater samples were collected from Vestfjorden Avløpselskap (VEAS), the Oslo wastewater treatment plant (WWTP) in June 2016. A total of 10 flow proportional samples were collected with an EFCON® Wall Mounted Vacuum sampler from the VEAS raw inlets between the 17th and the 30th of June. The sampler was operated at 4 °C and the wastewater samples were firstly collected in high-density polyethylene (HDPE) bottles and then homogenized, poured into the 7 mL glass vials and stored at −20 °C immediately following collection.

Weekend composite samples consisted of a three-day composite sample from Friday (08:00) to Monday (08:00) while weekdays were twenty-four-hour composite samples. VEAS treats sewage for a *de jour* population of approximately 600,000 people of which the city contributes about 70.5% and the adjoining areas representing the other 29.5%. The total length of the sewer line is 42.3 km and the mean residence time in the sewer system is 5 h [23].

2.3. Sample preparation and μ-SPE

Sample preparation is a crucial step to remove any matrix components that may compete with the target analytes in the ionization process during the UHPLC-MS/MS analysis. Prior to extraction, 5 mL of influent wastewater were spiked with 50 μl of the ILIS working solution to reach a concentration of 100 ng L^{-1}. Following vortex stirring, 1 ml of sample was centrifuged at 16,200 × for 5 min at 4 °C in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA, USA) and the supernatant was used for analysis. μSPE was performed using Waters Oasis HLB μElution plates, 30 μm (Milford, MA, USA). The plate was conditioned by washing and rinsing with 1 ml of MeOH and 1 ml of ultrapure water under suction. The wastewater samples were loaded onto the plate under suction and washed with 1 ml of ultrapure water. The plate was vacuum dried for 15 min. Analytes were eluted into a 96 well plate using 50 μl of 1% NH_4OH in MeOH, 100 μl of MeOH and 50 μl of 1% FA in MeOH.

The final 200 μl extract was divided in two LC vials for separate analysis for both target and retrospective purposes (Fig. 1). No solvent evaporation or residue re-dissolution were needed before injection and therefore, the eluent consisted only of methanol. Analysis was performed by injecting 37 μl into the PLM-LVI-UHPLC-MS/MS.

2.4. LC–MS/MS analysis

Wastewater analysis was carried out with a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager and a sample manager. The UHPLC was coupled to a Waters Quattro Premier XE Micromass triple quadrupole mass spectrometer (Milford, MA, USA) with a T-wave collision cell and electrospray ionization interface (ESI), operated in positive ionization mode. Selected parent and product ions together with ionization and collision energy parameters are presented in Table 1. Mass spectrometer parameters were tuned with a direct infusion of standard solutions. Information about the HRMS acquisition parameters and other information can be found in Baz-Lomba et al. [24].

Chromatographic separation was carried out using a Waters Acquity UPLC BEH C8 column, 1.7 μm, 2.1 × 100 mm (Milford, MA, USA). The column temperature was kept at 50 °C and the temperature of the sample manager was 4 °C. A constant flow rate of 0.4 ml min^{-1} was used with a mobile phase consisting of 0.1% ammonium hydroxide (solvent A) and acetonitrile (solvent B). The elution gradient changed as follows: 0 min (3% B); 4.9 min (3% B); 5.1 min (40% B); 8.5 min (60% B); 9 min (95% B); 10 min (95% B); 10.5 min (3% B); 11 min (3% B). The sample injection volume was 37 μl.

The cone and desolvation gas used was nitrogen with flow rates of 50 L h^{-1} and 800 L h^{-1}, respectively. The collision gas used was argon with a flow rate of 0.15 mL min^{-1}. Other operational parameters were capillary voltage, 3.2 kV; source temperature, 100 °C and desolvation temperature, 450 °C. The loop and needle volumes were 50 and 250 μl respectively and the injection mode was partial loop with needle overfill mode (PLNO). The PLNO mode provides the best partial loop accuracy, precision, and linearity and only sample and mobile phase were injected onto the column avoiding air gaps or weak wash solvent.

Data acquisition was performed working in multiple reaction-monitoring mode (MRM). Infusion solutions of individual standards were prepared to optimize MS conditions and to select MS/MS transitions for both target analytes and ILIS. The best results in terms of sensitivity were those using ESI operating in positive ionization mode, using the protonated molecule $[M+H]^+$ as precursor ion. The most abundant product ion of each target analyte was typically used for quantification and one additional product ion was used for confirmation. Furthermore, the retention times were also compared with those from reference standards (0.2 min). Each DTR was quantified using its ILIS as a surrogate internal standard, except citalopram, carbamazepine and methylphenidate for which the ILIS with the most similar retention time and chemical structures were selected. All data were acquired and processed using MassLynx v4.1 (Milford, MA, USA).

2.5. Method validation

Method validation was performed in terms of linearity, method quantification limits (LOQ), relative and absolute recoveries (trueness), repeatability and matrix effects. The performance of the method was evaluated following EU guidelines with minor modifications [25]. The linearity of the method was studied by analyzing standard solutions in methanol in triplicate at eight concentrations, in the range of 0.025–10 ng mL^{-1}, together with the ILIS at 0.5 ng mL^{-1}. Satisfactory linearity was considered when the correlation coefficient (R^2) was higher than 0.99, based on relative responses (analyte peak area/ILIS peak area). The LOQs were calculated in wastewater samples with known concentrations (all compounds were present in sample) as the concentrations giving a signal-to-noise ratio (S/N) of ≥10.

Relative and absolute recoveries were tested in triplicate in wastewater samples spiked at 100 ng L^{-1}. Adequate blank samples were not found since the target compounds were present in all the wastewater samples. Therefore, an additional set of three wastewater samples were analyzed by spiking only the ILIS before extraction to account for the analyte background. Relative recoveries were calculated by spiking the ILIS before the μSPE while for the absolute recoveries, meant for the assessment of the μSPE efficiency, were spiked after the extraction, right before the injection in the LC–MS/MS system. Calibration standards in solvent were used for quantification and the relative recoveries between 80% and 120% were considered satisfactory. Precision (expressed as repeatability) was assessed as the relative standard deviation (RSD) of six wastewater samples spiked at 200 ng L^{-1}. The matrix effects that occurred during the ionization (ESI) were assessed by spiking three wastewater extracts at 1 ng mL^{-1} together with the respective ILIS right before analysis and comparing its responses with that for those spiked at the same concentration in mobile phase. A non-spiked wastewater sample (only with ILIS) was analyzed simultaneously to subtract its response from the spiked sample:

$$Matrix\ effect\ (\%) = \frac{Response\ in\ ww\ extract - Response\ ww\ blank}{Response\ in\ mobile\ phase}\ x\ 100$$

3. Results and discussion

3.1. Large volume injection and post-loop mixing injection

The SPE extract is commonly evaporated under a current of nitrogen and reconstituted into the initial mobile phase to improve the chromatographic separation and avoid the sample to significantly penetrate the column without an optimal retention [24]. The PLM configuration, described in Fig. 2, avoids the eluent reconstitution following μSPE and chromatographic peak distortion when using LVI with UHPLC. In the PLM-LVI configuration, the position of the mixer and line A (aqueous phase) are changed in such a way that line B (organic phase, acetonitrile) goes directly to the loop in the autosampler, drags the sample and meets the aqueous phase in the mixer located right after the autosampler and before the HPLC column. At this stage, the sample is diluted in the mixer and stacked at the head of the column. Furthermore, the PLM-LVI configuration mitigates one of the main issues when using LVI with

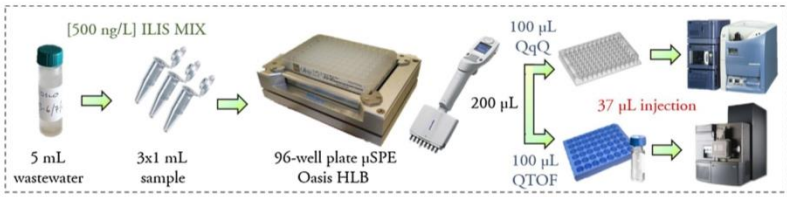

Fig. 1. Illustrative workflow of the analytical procedure using Waters Oasis HLB μElution plate (5 mg, 30 μm particle size) for the sample extraction (n = 3) and an UPLC-MS/MS for target quantification and UPLC-HRMS for virtual library bank acquisition.

Table 1
MS/MS optimized conditions for selected compounds.

Compound	ESI	Retention time	Quantitation MRM (Q1 > Q3)	Cone (V)	Collision (V)	Confirmation MRM (Q1 > Q3)
Amphetamine	+	6.6	136.1 > 91.1	20	15	136.1 > 119.1
Amphetamine-d8	+	6.6	144.1 > 97.1	20	15	–
Methamphetamine	+	7.0	150.1 > 91.1	20	15	150.1 > 119.1
Methamphetamine-d11	+	7.0	161.2 > 127.1	20	15	–
MDMA	+	6.9	194.2 > 163.2	20	15	194.2 > 105.1
MDMA-d5	+	6.9	199.2 > 165.2	20	15	–
Cocaine	+	7.4	304.2 > 182.2	30	20	304.2 > 105
Cocaine-d3	+	7.4	307.2 > 185.2	30	22	–
Benzoylecgonine	+	5.9	290.2 > 168.2	30	20	290.2 > 105
Benzoylecgonine-d3	+	5.9	293.2 > 171.2	30	20	–
Cocaethylene	+	8.0	318.2 > 196.2	30	20	318.2 > 82.1
Cocaethylene-d3	+	8.0	321.2 > 199.1	30	20	–
Atenolol	+	6.0	267.2 > 190	25	20	267.2 > 145
Atenolol-d7	+	6.0	274 > 145	30	20	–
Citalopram	+	8.1	325.2 > 262.2	30	22	325.2 > 109.2
Carbamazepine	+	6.5	237.1 > 194.1	25	20	237.1 > 192.1
Fexofenadine	+	5.9	502.3 > 466.3	20	30	502.3 > 171.1
Fexofenadine-d6	+	5.9	508.3 > 472.5	30	30	–
Methylphenidate	+	7.2	243.3 > 84	20	20	243.3 > 174.1
Metoprolol	+	6.7	268.2 > 116	25	20	268.2 > 191
Metoprolol-d7	+	6.7	275.2 > 123.1	28	20	–
Lidocaine	+	7.7	235.3 > 86	25	20	235.3 > 58.1
Lidocaine-d6	+	7.7	241.3 > 86	25	15	–

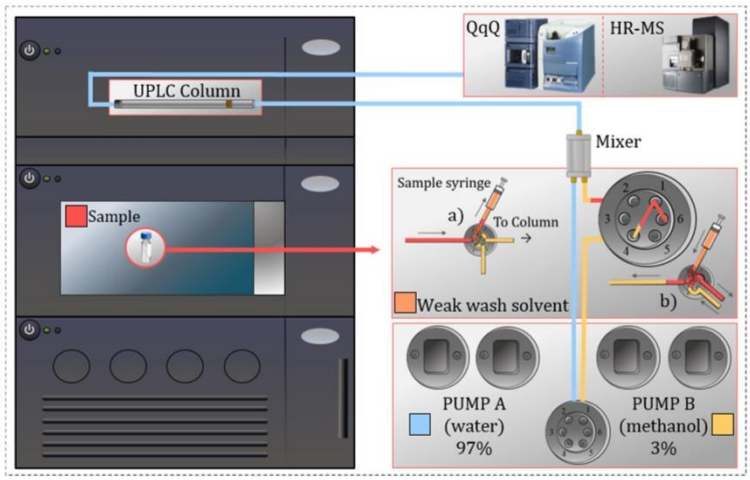

Fig. 2. Schematic representation of post-loop mixing process. Initial mobile phase rate (flow 0.5 mL min^{-1}) is set at 97% A:3% B during the first 5 min in order to retain the sample at the head of the UHPLC column.

UHPLC columns related to the lower sample capacity leading into chromatographic distortions such as peak broadening or volume over-load issues. By using a high initial water ratio (i.e. 97%), the sample is completely diluted in water right before the UHPLC column and retained in a narrow band at the head of the column.

The ratio of the organic phase *versus* aqueous phase will depend on the characteristics of the target compounds and becomes a critical feature in the development of the method. Optimization of the percentages of organic phase (acetonitrile) in water was achieved by comparing the peak shapes of the early-eluting compounds. The initial gradient was tested at 1–5 and 10% of acetonitrile. If the initial ratio of acetonitrile was too high, the polar analytes could not be retained at the column head due to the strong elution strength and therefore, the peak width of the analytes increased significantly. Both the loop and tubing (from autosampler to mixer) volumes were taken into account to estimate the time to fill the loop and drag the sample into the column (approximately 60 μL). The best compromise between peak shape and total run time was found to be 3% acetonitrile in water. Using a flow rate of 0.4 ml min^{-1}, the initial gradient was held for 5 min at 3% acetonitrile and once the analytes were retained at the head of the column the % acetonitrile was increased.

3.2. Method validation

The principal aim of this study was to prove the concept and applicability of a μSPE- PLM-LVI -UHPLC-MS/MS configuration for WBE. Therefore, neither the μSPE nor the UHPLC conditions were optimized. However, all the conditions and parameters used in this study were previously developed "in-house" for validated and published methods [24,26]. Furthermore, the analytical method used in this study has been validated through an external inter-laboratory exercise with other 27 international laboratories for some of the studied compounds (cocaine, benzoylecgonine, amphetamine, methamphetamine and MDMA), successfully meeting all the external quality control requirements [27].

The mean correlation coefficients (R^2) of the calibration curves, which are higher than 0.99 (Table 2) show good linearity of the method in the range of 0.025–10 ng mL^{-1}. The method LOQs were below 10 ng L^{-1} for all the compounds, ranging from 0.2 ng L^{-1} for carbamazepine to 6.3 ng L^{-1} for MDMA, being better than achieved with and SPE-UHPLC-MS/MS method on the same 16-year old MS system [26] and were below the reported environmental concentrations of the target compounds in wastewater.

The absolute recoveries for the μSPE performed with Waters Oasis HLB were satisfactory with values higher than 79% for all the compounds except for amphetamine with only a 36% recovery. Satisfactory relative recoveries were found for all the compounds, ranging from 92% for citalopram to 110% for cocaine. Precision (n = 6) for spiked wastewater samples at 200 ng L^{-1} was satisfactory in all cases with RSD values ranged from 3.4–14.4%.

3.3. Matrix effects

Ion suppression or enhancement is commonly observed in complex environmental matrices such as wastewater as a consequence of the matrix effect, which affects sensitivity, accuracy and the evaluation of method recovery. The matrix effect observed for the target compounds dissolved in wastewater is presented in Table 2. Little or no signal suppression was observed for MDMA, citalopram, carbamazepine and metoprolol. Atenolol and fexofenadine, both co-eluting at the beginning of the chromatographic run, showed a high ion suppression while the rest of the compounds showed a moderate ions suppression/enhancement (20%). The matrix suppression and recoveries were acceptable for the compounds for which no corresponding isotope-labelled internal standards were available.

3.4. Analysis of wastewater samples

The developed method was applied to the analysis of ten 24-h flow proportional influent samples (72-h for the weekend samples). Standard calibration curves were used to calculate the concentrations of the target compounds and injected in duplicate at the beginning and at the end of the run. Fortified "blank" samples were injected as internal quality control during the sequence.

The target compounds were found in all the inlet wastewater samples with changing concentrations (Table 3). Carbamazepine showed the highest concentrations with an average (n = 10) of 1200 ng L^{-1} while cocaethylene and metoprolol showed the lowest concentrations with an average of 9 and 7 ng L^{-1} respectively. Amphetamine and methamphetamine concentrations show similar levels ranging from 200–600 ng L^{-1} respectively. MDMA was the compound with the highest coefficient of variance among the 10 samples (61%) when comparing week days with the weekend due to its recreational use during the weekend in agreement with previous works [28]. Cocaine and its main metabolite, benzoylecgonine, ranged from 100–700 ng L^{-1} and show a benzoylecgonine/cocaine ratio of approximately 2–3, in agreement with previous publications [12]. For the rest of the pharmaceuticals, concentrations ranged from 25–48 ng L^{-1} for atenolol, from 35–71 ng L^{-1} for citalopram, from 117–205 ng L^{-1} for fexofenadine, from 140–263 ng L^{-1} for methylphenidate and from 55–108 ng L^{-1} for lidocaine.

3.5. Environmental feasibilities and implications for the future

In summary, the 96-well plate for μSPE provides the highest throughput for the analysis of wastewater samples to date. The main advantages are the reduction of the time invested per sample, the final cost per sample is lower (only the cartridges are approximately 25% less expensive and the amount of ILIS used compared with classic methods is approximately 100 times less), slightly decrease of matrix effects due to the reduction of the volume extracted and from the environmental point of view, is more feasible due to the reduction of the solvents used for the extraction, by approximately a 90%.

Furthermore, the HLB sorbent, with a hydrophilic-lipophilic-balanced sorbent, offers the possibility to extract a wide range of compounds with different psychochemical characteristics enabling the simultaneous analysis of a wide range of drugs and pharmaceuticals in one single extraction. The use of this generic extraction methodology also is very suitable for HRMS and retrospective analysis, which have been proposed as a good alternative for data storage and environmental repository without the need of additional sample analyses [29]. Furthermore, this workflow does not imply additional extractions and both analysis are performed using the same extract. Therefore, the approach proposed in Fig. 1 will allow the performance of different tasks such as pre- and post-target analysis, potential elucidation of metabolites and transformation products, retrospective analysis and non-target analysis only with one extraction and two analysis.

The PLM-LVI configuration complements the μSPE reducing even more the sample preparation time by avoiding the reconstitution of the eluent. Furthermore, this configuration also improves the efficiency of the method by injecting larger volumes. Most of the published analytical methods for the analysis of wastewater samples reconstitute the eluent in 250–1000 μL for a final injection of a few μL (normally between 2–5 μL) [24,30]. In this study, we elute 200 μl that are split in two for target and retrospective analysis, and 37 μl out of 100 μl are injected into the system. This configuration would also allow the introduction of robots or automated

Table 2
Method performance parameters: linearity, recoveries, repeatability, matrix effect and method limits of quantification.

	MeOH	Wastewater					ILIS used for correction
	Linearity (R^2) ng mL^{-1} n = 3	Relative recovery (RSD) Both in % [100 ng L^{-1}] n = 3	Absolute recovery (RSD) Both in % [100 ng L^{-1}] n = 3	Repeatability (RSD) % [200 ng L^{-1}] n = 6	Matrix Effects % n = 3	LOQ ng L^{-1}	
Amphetamine	0.025–10 (0.99931)	105 (14)	36 (18)	14.4	80	3.5	Amphetamine-d8
Methamphetamine	0.025–10 (0.99941)	94 (10)	95 (3)	9.3	117	1.1	Methamphetamine-d11
MDMA	0.025–10 (0.99973)	99 (3)	86 (5)	3.5	104	6.3	MDMA-d5
Cocaine	0.025–10 (0.99991)	110 (8)	79 (1)	6.8	117	4.3	Cocaine-d3
Benzoylecgonine	0.025–10 (0.99979)	103 (5)	86 (14)	4.3	87	2.9	Benzoylecgonine-d3
Cocaethylene	0.025–10 (0.99997)	98 (3)	86 (1)	3.4	118	1.0	Cocaethylene-d3
Atenolol	0.025–10 (0.99871)	104 (12)	87 (3)	11.1	55	4.4	Atenolol-d7
Citalopram	0.025–10 (0.99984)	92 (10)	87 (7)	11.3	96	1.1	Cocaethylene-d3
Carbamazepine	0.025–10 (0.99937)	104 (9)	93 (15)	11.3	102	0.2	Metoprolol-d7
Fexofenadine	0.025–10 (0.99980)	96 (8)	90 (12)	8.8	21	5.6	Fexofenadine-d6
Methylphenidate	0.025–10 (0.99979)	105 (7)	91 (11)	4.9	78	1.9	Cocaine-d3
Metoprolol	0.025–10 (0.99954)	109 (16)	94 (3)	12.6	104	2.1	Metoprolol-d7
Lidocaine	0.025–10 (0.99989)	100 (3)	92 (5)	3.6	113	0.3	Lidocaine-d6

Table 3
Concentrations of the target compounds quantified in 10 wastewater samples from Oslo in 2016 (ng L^{-1}).

Wastewater Concentration (ng/L)										
Date Compound	17–19/06/16 Weekend	20/06/16 Monday	21/06/16 Tuesday	22/06/16 Wednesday	23/06/16 Thursday	24-26/06/16 Weekend	27/06/16 Monday	28/06/16 Tuesday	29/06/16 Wednesday	30/06/16 Thursday
Amphetamine	459	282	227	372	426	594	393	372	402	349
Methamphetamine	447	300	250	375	398	480	386	353	395	354
MDMA	117	61	28	45	44	145	78	44	38	35
Benzoylecgonine	644	340	236	405	535	718	495	420	456	371
Cocaine	300	119	108	195	257	306	187	194	195	176
Cocaethylene	16	6	4	7	10	16	9	8	9	8
Atenolol	42	30	25	39	44	38	33	43	48	32
Citalopram	65	58	35	60	66	59	58	66	71	55
Carbamazepine	1379	1241	888	1315	1433	1277	1168	1200	1389	1091
Fexofenadine	205	165	117	167	178	182	142	166	165	165
Methylphenidate	185	167	140	205	263	204	215	232	232	177
Metoprolol	6	5	3	7	10	9	7	7	7	5
Lidocaine	89	87	55	71	85	83	72	77	108	78

μSPE that would simplify and improve substantially the method in the future.

The reduction of the time invested for collecting, extracting and analyzing the sample together with substantial reduction of the cost, increase the possibilities for the laboratories to perform real-time monitoring. The fact that the staff at the WWTP move from collecting 500–1000 ml in big plastic bottles to 5 mL in small glass vials could potentially increase the number of collaborations between laboratories and treatment plants.

4. Conclusions

A novel analytical methodology based on the use of μSPE-PLM-LVI-UHPLC-MS/MS has been developed for the simultaneous quantification and confirmation of 13 widely consumed drugs in urban wastewater and applied to 10 influent wastewater samples from Oslo, Norway. A high throughput analytical procedure has been fully validated, obtaining satisfactory accuracy and precision and high sensitivity. The method LOQs are comparable with previous studies and below the environmental concentrations found is Oslo during the last years.

The combination of μSPE with PLM-LVI has been demonstrated to be a promising compromise to reduce the sample preparation time and still reach the required detection levels for environmental samples. Furthermore, reducing total cost and amounts of solvents, increasing the method efficiency and improving the collection and handling of the samples, have upgraded the technical and environmental feasibility of classic WBE methods. These results highlight the potential of μSPE-PLM-LVI-UHPLC-MS/MS for WBE studies in the future.

In addition, a 200 μl μSPE extract is enough for both the quantitative and HRMS analysis, which will enable the creation of a virtual specimen library bank for WBE. This additional workflow will archive all the data for retrospective analysis, functioning as a backup for cases when old samples are not available or degraded.

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Paper III

Illicit drug use in Reykjavik by wastewater-based epidemiology

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Abstract

Estimation of illicit drug use on a community level by wastewater-based epidemiology (WBE) is both an objective and reliable way to establish near real-time results. Wastewater samples were collected at eleven timepoints in Reykjavik from 2017 to 2020. Commonly abused illicit drugs in Iceland (amphetamine, methamphetamine, 3,4 methylenedioxymethamphetamine (MDMA), cocaine and cannabis) were analyzed using solid phase extraction and ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Estimated amphetamine and methamphetamine use showed signs of an increase from 2017 to 2020 with amphetamine being the dominant stimulant on the market. Estimated MDMA use remained stable from 2017 to 2020. Results showed a large increase in cocaine use from 2017 to 2019 but interestingly, a marked decrease in 2020 during the COVID-19 pandemic. Estimated cannabis use was stable from 2017 to 2019 but showed signs of an increase during the pandemic in 2020. Results by WBE corresponded with data based on other indicators of drug use, seizure data and driving under the influence cases. Both temporal and spatial trends in illicit drug use were successfully estimated by using WBE, complimenting other indicators which provided a comprehensive picture of drug abuse in Reykjavik.

Keywords

Wastewater-based epidemiology; drug consumption; illicit drugs; drug seizures, driving under the influence

1. Introduction

Availability and purity of illicit drugs is on the rise in Europe. This will result in harmful effects on human health and social welfare with increasing numbers of drug-related deaths (European Monitoring Centre for Drugs and Drug Addiction, 2019; United Nations Office on Drugs and Crime, 2018). WBE has provided insight into the extent of illicit drug consumption since first applied in 2005 (Zuccato et al., 2005). Parent compounds and metabolites of illicit drugs are excreted into the wastewater system and reach wastewater treatment plants (WTP). The wastewater can be considered a pooled urine sample of a population, resided in a defined area (Daughton, 2001; Zuccato et al., 2005). This methodology effectively monitors both temporal and spatial trends in drug use with objective and rapid estimations (Castiglioni et al., 2014). WBE has shown clear advantages over other indicators of illicit drug use with known biases such as traditional survey methods (Castiglioni et al., 2014; Kankaanpää et al., 2016). Nevertheless, there are uncertainties associated with the use of WBE such as limited data on excretion rates used in the determination of consumed amounts. It is important to use excretion rates that represent the most common routes of administration for the relevant population, if available (Gracia-Lor et al., 2016). Other known uncertainties are associated with e.g. the assessment of the population size behind a sample and sampling techniques (Castiglioni et al., 2013).

Since 2015, WBE has been used to investigate the stimulant drug market in Iceland. Results have shown high levels of amphetamine in wastewater compared to other European countries and recently a large increase in cocaine levels (González-Mariño et al., 2020; Löve et al., 2018). By only using WBE, it is however not possible to determine if these trends are due to changes in the number of drug users, number of consumed doses or changes in drug purity (Bruno et al., 2018). A more comprehensive picture of illicit drug use can be provided by complementing WBE with other indicators of drug use. These are, e.g. driving under the influence (DUI) cases and data on seized amounts of drugs (Bruno et al., 2018; Kankaanpää et al., 2016).

Until now, information on illicit drug use in Iceland has mainly been obtained through self-reported consumer interviews and medical figures from rehabilitation centers (The Directorate of Health, 2012; Tyrfingsson, 2019). The National Centre of Addiction Medicine (SÁÁ) in Iceland aims to determine the most problematic substance for patients entering drug rehabilitation. As of 2015, cannabis was most frequently problematic for patients, followed by amphetamine and cocaine (Tyrfingsson, 2019). A survey on illicit drug use in

Iceland conducted in 2018 (1,277 individuals, aged 18 to 67) showed similar results with cannabis being most frequently used followed by amphetamine and cocaine. (Kristjánsson and Jónsson, 2019). According to a descriptive population-based study on intravenous use in patients seeking treatment, methylphenidate was the drug of choice followed by opioids, amphetamine and cocaine (Bjarnadottir et al., 2015). The number of intravenous users has increased along with a rising number of patients in rehabilitation centers (Bjarnadottir et al., 2015; Tyrfingsson, 2019). Organized crime groups in Iceland handle most import, production, sales and distribution of illicit drugs (National Police Commissioner of Iceland, 2019). An increase in cocaine import is considered an indication of a rising number of these crime groups in Iceland (National Police Commissioner of Iceland, 2019). Now with record breaking numbers of cocaine seizures in Europe, high availability of the drug has led to a considerable increase in purity (European Monitoring Centre for Drugs and Drug Addiction, 2020). This poses a risk to users that are accustomed to lower doses, leading to an increase in drug related deaths and rising numbers of users who seek help for cocaine dependence (National Police Commissioner of Iceland, 2019; Tyrfingsson, 2019). With higher availability and purity of drugs with rising numbers of users, it is important to closely observe new trends in illicit drug use so appropriate preventative actions can be taken.

For the first time, this study aims to compare illicit drug use by WBE with statistics on positive DUI cases and seized amounts of drugs by the police. Also, results on tetrahydrocannabinol (THC) in Icelandic wastewater have not been reported before. The objective is to provide a clearer picture on the extent of illicit drug use in Iceland and determine trends in use.

2. Materials and methods

2.1 Chemicals and materials

Amphetamine hydrochloride, benzoylecgonine (BE), cocaine hydrochloride, methamphetamine hydrochloride, MDMA hydrochloride and d,l-11-Nor-∆9-tetrahydrocannabinolic-carboxylic acid (THCA) were purchased from Lipomed (Arlesheim, CH). Corresponding isotope-labeled internal standards (ILIS) amphetamine-d3 and cocaine-d3 were purchased from Lipomed (Arlesheim, CH). Benzoylecgonine-d3, MDMA d5 and methamphetamine d5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetrahydrocannabinolic acid-d3 was purchased from Cerilliant (Round Rock, TX, USA). Reference

standards and internal standards were in methanol (MeOH), ethanol (EtOH) or acetonitrile (ACN) at concentrations of 1 mg/mL or 100 µg/mL. MeOH and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ACN was from Honeywell (Charlotte, NC, USA). Water was purified using a Milli-Q Integral 3 water purification system. Oasis HLB 3cc Vac solid phase extraction (SPE) cartridges (60 mg sorbent, 30 µm particle size) were purchased from Waters (Milford, MA, USA).

2.2 Sample collection and storage

Sample collection was carried out from two WTPs, Skerjafjarðarveita and Sundaveita, that collect wastewater from approximately 80% of the population in the Reykjavik metropolitan area. These WTPs both remove coarse material (fat and sand) from the wastewater before it is released into the ocean. 24h samples were collected in time-proportional mode over seven days in 2017 (February, March, May, July, August, October, and November), 2018 (January and March), 2019 (April), and 2020 (June). Sample collection in 2019 and 2020 was only conducted in Sundaveita WTP.

2.3 Sample extraction procedure

A SPE procedure for sample preparation was based on Bijlsma et al. (Bijlsma et al., 2014) and carried out as follows: Oasis HLB cartridges were conditioned with 6 mL of MeOH and 6 mL of purified water. 50 mL aliquots of wastewater samples were centrifuged after the addition of ILIS (180 ng/L). The supernatant of the wastewater sample was then loaded onto the cartridges. The cartridges were washed with 3 mL of purified water after loading and vacuum dried for 15 min. The analytes of interest were then eluted off the cartridges with 5 mL of MeOH. The eluate was evaporated at 40°C to dryness and the residue was reconstituted in 1 mL MeOH. A 50 µL aliquot of the final MeOH extract was further diluted in 450 µL of purified water. 5 µL of both mixtures were then injected into the ultra-high performance liquid chromatography tandem mass spectrometry system (UHPLC-MS/MS).

2.4 Instrumental conditions

Instrumental analysis was performed using Waters Acquity UPLC I-Class system, consisting of a column manager, sample manager (fixed loop) and a binary solvent manager. A Waters Acquity UPLC® BEH C18 column, 1.7 µm,

2.1×100 mm (Milford, MA, USA) was used for chromatographic separation with a column temperature of 40°C. A constant flow rate of 0.4 mL/min was held with a mobile phase consisting of 0.1% formic acid (solvent A) and ACN (solvent B). The gradient was as follows: 2% B from 0 to 1.5 min, 13% B at 1.8 min, 36% B at 2.65 min, 50% B at 3.4 min, 95% B at 3.45 min, 2% B at 2.8 min.

The UPLC system was coupled to a Xevo TQ-S micro mass spectrometer with a step wave ion guide and electrospray ionization (ESI). The cone gas was nitrogen with a gas flow rate of 20 L/h, the desolvation gas was nitrogen with a gas flow rate of 800 L/h and the collision gas was argon. Capillary voltage was set to 1 kV. The source temperature and desolvation temperature was 150°C and 500°C, respectively. Selected quantifier and qualifier transitions along with other MS/MS parameters for each compound are presented in Table 1.

Table 1

Selected quantifier and qualifier transitions along with other MS/MS parameters for the selected analytes.

	ESI polarity	**Quantifier transition (Q1 > Q3)**	**Cone / Collision (V / V)**	**Qualifier transition (Q1 > Q3)**	**Cone / Collision (V / V)**
Amphetamine	+	136 > 119	25 / 15	136 > 90.9	25 / 7
Amphetamine-d3	+	139 > 92.0	25 / 15	-	-
Methamphetamine	+	150 > 119	35 / 16	150 > 90.9	35 / 10
Methamphetamine-d5	+	155 > 92.0	27 / 15	-	-
MDMA	+	194 > 163	36 / 23	194 > 105	36 / 11
MDMA-d5	+	199 > 165	32 / 10	-	-
Cocaine	+	304 > 82.0	38 / 28	304 > 182	38 / 18
Cocaine-d3	+	307 > 185	38 / 18	-	-
Benzoylecgonine	+	290 > 168	45 / 27	290 > 82.0	45 / 18
Benzoylecgonine-d3	+	293 > 171	38 / 18	-	-
THCA	-	343 > 245	60 / 26	343 > 299	60 / 18
THCA-d3	-	346 > 302	60 / 18	-	-

2.4 Method validation

The methodology was validated before analysis according to guidelines published by the European Medicines Agency (EMA), the International Union of Pure, Applied Chemistry (IUPAC) and the Scientific Working Group for Forensic toxicology (SWGTOX) with appropriate modifications (European Medicines Agency, 2011; International Union of Pure and Applied Chemistry, 2002; Kruve et al., 2015a, 2015b; Scientific Working Group for Forensic Toxicology, 2013). A detailed description of validation experiments and results can be found in the supplementary information.

2.6 Calculations

Daily mass loads (mg/day) of each analyte in wastewater were calculated per 1000 inhabitants. The mass loads were obtained by multiplying the measured concentration (ng/L) of each analyte or their metabolite in wastewater by the average wastewater flow rate (L/day). The consumed amounts were obtained by multiplying the mass loads by a correction factor. The correction factors are based on excretion percentages in urine and molecular mass ratio between the parent compound and metabolite (if applicable). Correction factors used in the calculations are presented in Table 2 along with mean excretion rates and molecular mass ratios. The excretion rates are either based on the weighted mean for different routes of administrations or, if available, the most appropriate route of administration for the target population (Castiglioni et al., 2013; Gracia-Lor et al., 2016). The daily consumed amounts were finally divided by the population of each WTP, based on census data, and normalized by 1000 inhabitants (mg/day/1000 inhabitants). Population numbers were obtained from Statistics Iceland and are based on registered residences in different postal codes of the Reykjavik area for each year. Parameters used in the calculations of used amounts of illicit drugs are listed in Table S3 in the supplementary information.

Table 2

Correction factors for each analyte used in the back-calculations and parameters used to obtain them (mean excretion of drug target residue and molecular mass ratio).

Drug target residue	Mean excretion (%)	Route of administration	Molecular mass ratio	Correction factor
Amphetamine	36.1[a]	Oral	1.00	2.77[a]
Methamphetamine	39.3[a]	Intranasal	1.00	2.54[a]
MDMA	22.5[b]	Oral	1.00	4.40[a]
Benzoylecgonine	29.2[a]	*	1.05	3.59[b]
THCA	0.500[a]	Smoked	0.910	182[a]

* Weighted mean of excretion percentages for different routes of administrations.

[a] (Gracia-Lor et al., 2016)

[b] (Castiglioni et al., 2013)

2.7 Comparison with other indicators of drug use

In this paper, results by WBE are compared with data on DUI cases and seized amounts of drugs by the Icelandic police.

Data on both the number of positive and negative DUI samples from 2014 to 2020 were obtained from the Department of Pharmacology and Toxicology (DPT), University of Iceland, where all Icelandic DUI cases are handled. Data collection by the DPT is based on the type of analysis requested by the police. Requested analysis by the police is based on preliminary testing of amphetamines, cannabis, and cocaine. Amphetamines are analyzed as a package which included amphetamine, methamphetamine and MDMA.

Data on seized amounts of illicit drugs was provided by the National Commissioner of the Icelandic police. This data contained information on seized amounts of illicit drugs from the year 2014 to 2019. Seized amounts of amphetamine is presented in both grams (powder form) and milliliters (liquid form). Seized amounts of methamphetamine and cocaine is presented in grams (powder form). Seized amounts of MDMA in both grams and pieces (tablets) was provided. Seized amounts of MDMA tablets was transformed to grams by multiplying the average weight (mg) of tablets received for analysis at the DPT from 2010 to 2019 by seized amounts of tablets by the police,

divided by the average concentration of MDMA crystals received for analysis at the DPT from 2012 to 2019. Data on seized amounts of cannabis included the number of plants in active soil cultivation, grams of cannabis plants in the drying process, grams of marijuana ready to be sold on the drug market and grams of hash.

3. Results and discussion

3.1 Amphetamine

The route of administration of illicit drugs in Iceland is not well described especially in the case of amphetamine. Heavy consumption is likely through intravenous injection, whereas recreational consumers depend more on nasal or oral effects (Tyrfingsson, 2019). However, very little data on excretion rates of amphetamine for different routes of administrations is available except for the oral route (Gracia-Lor et al., 2016). Therefore back-calculations of amphetamine use in Reykjavik by WBE are based on excretion rates after oral consumption.

Results from the analysis of amphetamine in wastewater samples collected in Reykjavik at eleven different time points from February 2017 to June 2020 are presented in Fig. 1. Results showed a significant increase ($p < 0.05$, t-test) in amphetamine use by 61% (from 571 to 920 mg/day/1000 inhabitants). Amphetamine use was relatively stable from February 2017 to March 2018 but had increased significantly ($p < 0.05$, t-test) by 60% in April 2019 (from 555 to 888 mg/day/1000 inhabitants). Results from the analysis of wastewater samples collected during the COVID-19 pandemic in June 2020 showed similar levels as in April 2019 (921 mg/day/1000 inhabitants). At the time, restrictions were on gatherings and opening hours of nightclubs, restaurants, and cafés. These results indicate that there were minimal effects due to the pandemic on amphetamine use at that time. Overall, the data presented shows signs of increased use of amphetamine in Reykjavik. Previous studies on spatial trends have reported high amphetamine loads in wastewater from northern European cities, including Reykjavik, Oslo, Stockholm and Helsinki, compared to southern parts of the continent (González-Mariño et al., 2020; Löve et al., 2018). Results showed up to a 31% increase in amphetamine use during weekends (Saturday to Sunday). Although increased use of amphetamine during weekends was only considered significant ($p < 0.05$, t-test) in six out of eleven sampling periods, the results indicate that the drug is used recreationally to some extent. A music festival (Iceland Airwaves) was

held in Reykjavik during the November 2017 sampling period (from Thursday to Sunday). The results showed a 66% increase in amphetamine use during the music festival compared to other weekdays with a large spike on Friday (103% increase from Thursday) but had dropped again from Saturday to Sunday. These results indicate that amphetamine is used as a recreational drug during special events.

The number of positive DUI cases for commonly used illicit drugs from 2014 to 2020 is presented in Fig. 2. The number of positive DUI cases increased steadily by 117% from 2014 to 2019 (from 514 to 1120 cases) but decreased by 20% from 2019 to 2020 (from 1120 to 886 cases). The number of cases was relatively stable from 2014 to 2016 but increased by 81% from 2016 to 2019 with the largest leap between 2016 and 2017 (43% increase). The total number of cases where the analysis of amphetamines was requested by the police increased by 92% from 2014 to 2019 but had decreased by 27% in 2020. The percentage proportion of positive samples compared to the total number of DUI cases each year remained relatively stable for the seven-year period which indicates that the criteria for requested analysis remained the same throughout the seven-year period. These results suggest that the total number of amphetamine consumers is growing, resulting in an increased number of positive DUI cases. This supports data by WBE which also indicates increased amphetamine use. A rising number of patients in rehabilitation centers since 2014 further supports this trend (Tyrfingsson, 2019). The decrease in DUI cases in 2020 could be explained by the social disruption caused by the COVID-19 pandemic. Decreased mobility due to e.g. restricted opening hours of restaurants, cafés and nightclubs could have affected the number of DUI cases.

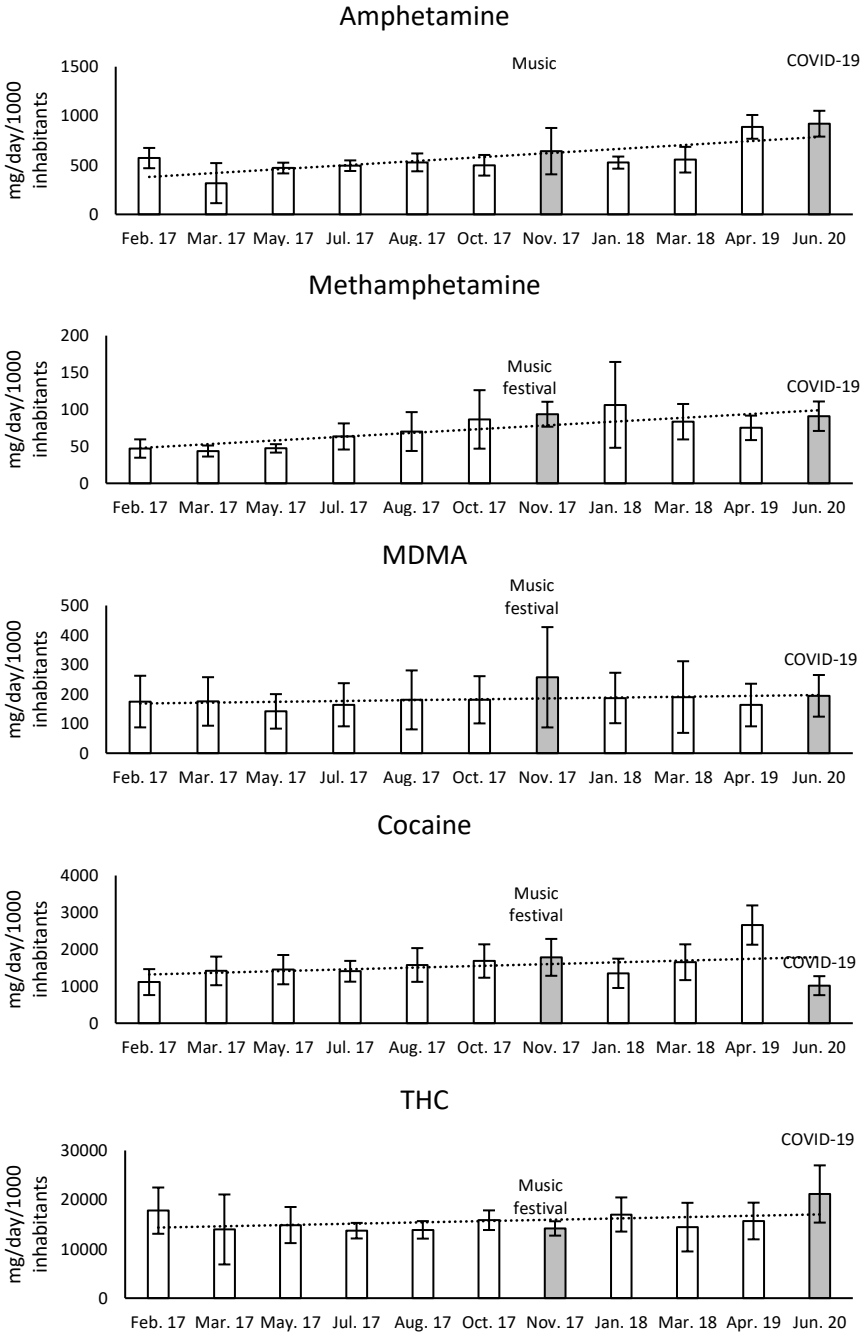

Fig. 1. Estimated use of amphetamine, methamphetamine, MDMA, cocaine and cannabis in Reykjavik in mg/day/1000 inhabitants during eleven weeklong sampling periods between February 2017 and June 2020.

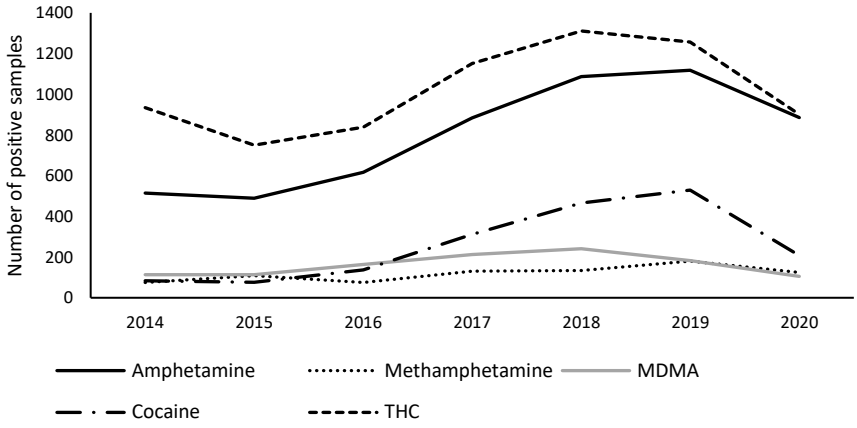

Fig. 2. The number of positive DUI cases for amphetamine, methamphetamine, MDMA, cocaine and cannabis in Iceland from 2014 to 2019.

Data on seized amounts of amphetamine from 2014 to 2019 in both powder and liquid form is presented in Fig. 3. Yearly data from the Icelandic police on seized amounts of amphetamine in powder form shows considerable fluctuations during the six-year period presented (2014 to 2019) with a range from 4.9 kg to 56 kg per year. It was not possible to detect similar trends in seized amounts of amphetamine powder compared to the number of positive DUI cases and data by WBE. Seized amounts of amphetamine in liquid form, amphetamine base intended for local production of amphetamine powder, also shows significant fluctuations over the six-year period. The total amounts of seized amphetamine base from 2014 to 2016 was negligible but increased greatly from 2017 to 2019. The extensive increase in seized amounts of amphetamine base in the past 3 years suggests that the production of amphetamine powder is on the rise in Iceland. Overseen by foreign criminal organizations, the supply of amphetamine in Iceland is stable indicating an abundance of amphetamine on the market (National Police Commissioner of Iceland, 2019). An increase in local production of amphetamine would support data by WBE and the number of positive DUI cases with an increasing number of amphetamine users and/or the concentration of the drug.

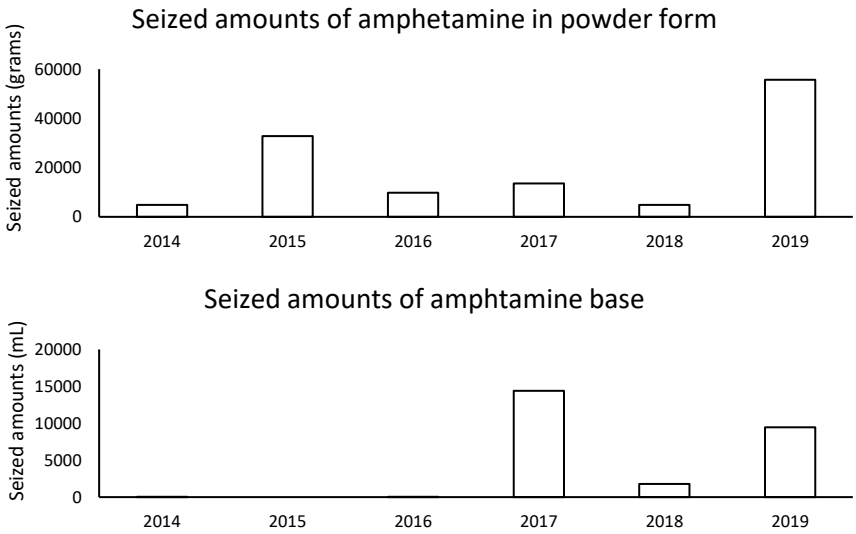

Fig. 3. Seized amounts of amphetamine in powder and liquid form by the Icelandic police from 2014 to 2019.

3.2 Methamphetamine

Results from the analysis of methamphetamine in wastewater samples collected in Reykjavik from February 2017 to June 2020 are presented in Fig. 1. Methamphetamine use according to WBE increased significantly ($p < 0.05$, t-test) by 94% during the total sampling period from February 2017 to June 2020 (from 47.1 to 90.9 mg/day/1000 inhabitants). A significant increase ($p < 0.05$, t-test) in methamphetamine use by 125% was observed from February 2017 to January 2018 (from 47.1 to 106 mg/day/1000 inhabitants), but amounts had dropped by 20% in March 2018. The results showed stable use from March 2018 to June 2020 indicating minimal effects caused by the COVID-19 pandemic. These trends could imply fluctuations in the Icelandic methamphetamine market where availability of the drug is variable. These results are in accordance with fluctuations in methamphetamine loads from other northern countries in Europe (González-Mariño et al., 2020). Traditionally, methamphetamine is not considered a staple of the Icelandic drug market and its use is low. Methamphetamine is mainly produced in Czechia which has led to high use of the drug in central Europe (European Monitoring Centre for Drugs and Drug Addiction, 2020; González-Mariño et al., 2020). Methamphetamine loads from Reykjavik have been reported to be

relatively low compared to other European countries (González-Mariño et al., 2020; Löve et al., 2018). Nevertheless, the results point to a rise in methamphetamine use which is in accordance with reports on increased availability of the drug in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2020). WBE can be a valuable tool in the early detection of changes in the local drug market by identifying rapidly emerging drugs such as methamphetamine (Gunnar and Kankaanpää, 2019). Trends between weekends and other weekdays in methamphetamine use varied considerably between sampling weeks ranging from no increase to 93% increase during the weekend. Methamphetamine use by WBE during the music festival held in November 2017 did not show significant trends compared to other normal weeks. These results indicate that methamphetamine is not used recreationally during weekends or special events to a large extent.

An increase by 140% was observed in the number of DUI cases positive for methamphetamine from 2014 to 2019 (from 75 to 180 cases) but decreased by 31% from 2019 to 2020 (from 180 to 125 cases) (Fig. 2). The percentage proportion of positive samples compared to the total number of DUI cases remained stable for the seven-year period. The decrease detected in 2020 is likely due to a reduction in commuting time during the COVID-19 pandemic. Nevertheless, these results support data by WBE that methamphetamine use is increasing in Iceland, although low compared to amphetamine.

Data on seized amounts of methamphetamine from 2014 to 2019 in powder form is presented in Fig. 4. In 2014 and the years before, seized amounts of methamphetamine powder remained almost non-existent. However, in 2015 seized amounts of methamphetamine began to increase, although still very low compared to amphetamine. Sporadic seizures in recent years, although small, could further indicate that the use of methamphetamine in Iceland is increasing, supporting data by WBE and DUI cases.

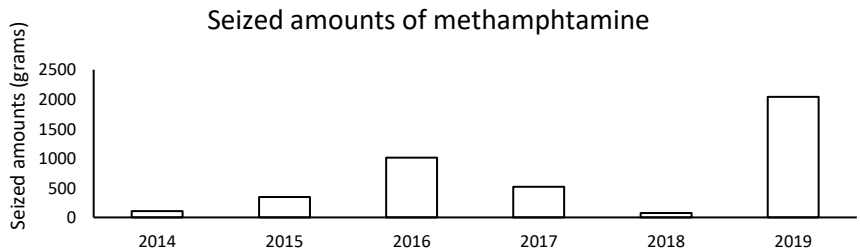

Fig. 4. Seized amounts of methamphetamine in powder form by the Icelandic police from 2014 to 2019.

3.3 MDMA

Results from the analysis of MDMA in wastewater samples collected in Reykjavik from February 2017 to June 2020 are presented in Fig. 1. Estimated MDMA use by WBE was close to constant during the total sampling period of this study (from 175 to 194 mg/day/1000 inhabitants). These results are consistent with other European studies based on prevalence data showing stable trends in MDMA use in recent years (European Monitoring Centre for Drugs and Drug Addiction, 2020). Nevertheless, an upsurge in MDMA loads in wastewater from other European countries have been observed which could be explained by increased purity of the drug (González-Mariño et al., 2020). In recent years, MDMA loads from Reykjavik have been in the medium range compared to other European cities (González-Mariño et al., 2020). MDMA use in June 2020 was stable compared to other sampling periods which indicates that the COVID-19 pandemic had not affected the amount of MDMA used in Reykjavik at that time point. MDMA use increased significantly ($p < 0.05$, t-test) during weekends compared to other weekdays in all sample periods, ranging from an increase of 42% to 154%. This strongly suggests that the drug is largely used recreationally which is in accordance with previously published studies (Löve et al., 2018; Ort et al., 2014; Thomas et al., 2012). An increase in MDMA use was also observed in November 2017 when a music festival was held in Reykjavik. MDMA use during the festival increased by 67% from Thursday to Sunday compared to other weekdays and 193% from Saturday to Sunday. These trends strongly indicate that MDMA is used as a recreational drug in Iceland during special events as well as weekends. These findings are supported by other European studies on the recreational use of MDMA during special events (Krizman-Matasic et al., 2019; Mackuľak et al., 2019).

The number of DUI cases with positive MDMA results increased by 61% from 2014 to 2019 (from 113 to 182 cases) but decreased by 42% from 2019 to 2020 (from 182 to 105 cases) (Fig. 2). These results show a steady increase between 2015 and 2018 but remained relatively stable from 2018 to 2019. The proportion of positive samples compared to the total number of cases were stable from 2014 to 2020. A probable cause for the decreased number of positive DUI cases detected in 2020 are the effects of the COVID-19 pandemic. Reduced opening hours of nightclubs and fewer special event may have led the drug use to take place in private homes with less commuting. These results are reasonably consistent with data by WBE which indicate stable MDMA use from the beginning of 2017.

Seized amounts of MDMA remained relatively low throughout the six year period from 2014 to 2019, presented in Fig. 5. Nevertheless, a significant peak in seized amounts was observed in 2015 but otherwise, negligible amounts of MDMA were seized from 2016 to 2019. Stable use of MDMA since 2017 according to data by WBE suggests that the drug is imported into the country without being discovered by the authorities.

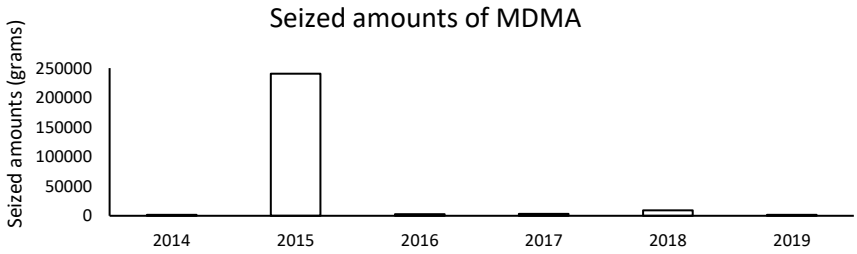

Fig. 5. Seized amounts of MDMA in powder form by the Icelandic police from 2014 to 2019.

3.4 Cocaine

Cocaine is excreted mostly as the metabolite benzoylecgonine. Consumed amounts of cocaine were calculated based on benzoylecgonine concentrations in wastewater. Results on cocaine use in Reykjavik from February 2017 to June 2020 by WBE are presented in Fig. 1. Cocaine use by WBE increased significantly ($p < 0.05$, t-test) by 139% from February 2017 to April 2019 (from 1110 to 2660 mg/day/1000 inhabitants). The largest increase was by 61% between March 2018 and April 2019 (from 1650 to 2660 mg/day/1000 inhabitants). These results indicate an upward trend in cocaine use in Reykjavik which is in accordance with other European reports on record breaking numbers of seizures (European Monitoring Centre for Drugs and Drug Addiction, 2020; González-Mariño et al., 2020). In recent years, cocaine loads from Reykjavik have been on the rise compared to loads from other European cities and are now in the upper range (González-Mariño et al., 2020). Increasing availability of cocaine in Europe has led to higher purity of the drug in Iceland as well as lower prices (European Monitoring Centre for Drugs and Drug Addiction, 2020; National Police Commissioner of Iceland, 2019). The effects of this rise in purity and availability of cocaine can be seen in increased numbers of patients in rehabilitation centers in Iceland due to cocaine dependencies (Tyrfingsson, 2019). The improving economic status in Iceland due to expanding tourism could be a contributing factor in the increased use of

cocaine as a similar trend was observed before the financial crash in Iceland in 2008 (Tyrfingsson, 2019). Estimated cocaine use by WBE in June 2020 during the COVID-19 pandemic had dropped significantly by 60% ($p < 0.05$, t-test) compared to April 2019 (from 2660 to 1020 mg/day/1000 inhabitants) which indicates a changed consumption pattern of the drug. All nightclubs in Reykjavik had restricted opening hours in June 2020 due to COVID-19 resulting in a shortage of the usual social gathering connected with recreational use of drugs. These different circumstances seem to have caused consumers to seek other substances or reduce use altogether. During all sampling periods, cocaine use by WBE increased over the weekend (from Saturday to Sunday) compared to other weekdays. This increase ranged from 29% to 68% during weekends apart from only a 10% increase in June 2020 during COVID-19. This increase was significant ($p < 0.05$, t-test) during all sampling periods except in June 2020. These trends between weekdays suggest that the drug is used recreationally during weekends which is consistent with previous reports (Löve et al., 2018; Ort et al., 2014; Thomas et al., 2012). A rise in cocaine use was also observed in November 2017 during the music festival when cocaine use increased by 55% (from Thursday to Sunday) compared to other weekdays. These findings further indicate recreational use of cocaine in Reykjavik during special events supporting other European studies (Krizman-Matasic et al., 2019; Mackuľak et al., 2019).

A more than six-fold increase was observed in the number of DUI cases positive for cocaine between the year 2014 and 2019 (from 83 to 529 cases) but a decrease was observed from 2019 to 2020 by 61% (from 529 to 208 cases) (Fig. 2). The number of positive samples was relatively stable from 2014 to 2016 (from 83 to 137 cases), but then began to increase to a great extent year by year from 2016 to 2019 (from 137 cases to 529 cases). The total number of cases where the analysis of cocaine was requested by the police increased more than 3-fold from 2014 to 2019 but had decreased by 36% in 2020. The decrease detected during the COVID-19 pandemic in June 2020 corresponded with data by WBE. The proportion of positive samples compared to the total number of cases remained relatively stable from 2014 to 2020. Overall, this data shows clear signs of increased cocaine use in Iceland.

Data on seized amounts of cocaine began to rise in 2014, reaching a maximum amount in 2019 with a 23-fold increase (from 1.7 kg to 41 kg), shown in Fig. 6. Along with the extensive rise in the number of positive DUI cases and cocaine amounts in wastewater, these results strongly indicate a large increase in cocaine use in Reykjavik, both in the number of consumers and/or the concentration of the drug. This could be explained by increased availability

of the drug and therefore decreased prices (National Center of Addiction Medicine, 2020; National Police Commissioner of Iceland, 2019).

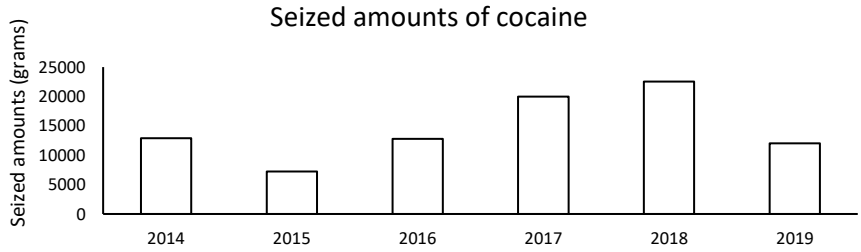

Fig. 6. Seized amounts of cocaine in powder form by the Icelandic police from 2014 to 2019.

3.5 Cannabis

THCA is the main metabolite of THC in urine, the active component of cannabis. Cannabis use was calculated based on THCA concentrations in wastewater. Results on cannabis use in Reykjavik from February 2017 to June 2020 by WBE are presented in Fig. 1. Cannabis use by WBE remained relatively stable from February 2017 to April 2019 (from 13.7 g/day/1000 inhabitants to 17.8 g/day/1000 inhabitants). These results correspond with reports on cannabis use in Europe amongst young people which have either shown stable or increasing trends in recent years (European Monitoring Centre for Drugs and Drug Addiction, 2020). A 35% increase ($p = 0.0573$, t-test) in cannabis use was observed in June 2020 during the COVID-19 pandemic (from 17.8 g/day/1000 inhabitants to 21.2 g/day/1000 inhabitants). This indicates a change in the consumption pattern of cannabis in Reykjavik during the pandemic with restricted opening hours of nightclubs and possibly increased consumption in private homes. Trends between weekdays did not show any significant changes between weekends and other weekdays from 2017 to 2019. No change in cannabis use was observed during the music festival held in November 2017. These results suggest that the use of products containing THC in Iceland is stable and points to daily use of the drug rather than recreational use. Low amounts due to recreational use could therefore be undetectable due much larger amounts being used daily. THCA has a very low excretion rate in urine which could also partly explain the lack of trends in the WBE data (Postigo et al., 2010). In June 2020 during the pandemic, cannabis use showed a significant increase by 42% ($p < 0.05$, t-test) during the

weekend. This further indicates a shift in the consumption patterns of illicit drugs during the COVID-19 pandemic where recreational use of stimulant drugs such as cocaine has been replaced to some extent with cannabis use in private homes. Nevertheless, it should be considered that only one 7-day sample collection was performed during the COVID-19 pandemic and therefore does not represent the total period of the pandemic.

Considerable fluctuations were observed in the number of DUI cases positive for THC from 2014 to 2020, shown in Fig. 2. The number of DUI cases decreased from 2014 to 2015 by 20% (from 934 to 750 cases), then increased steadily from 2015 to 2018 by 75% (from 750 to 1310 cases) but was stable from 2018 to 2019 (from 1310 to 1260 cases). In 2020, positive DUI cases decreased again by 28% (from 1260 to 901 cases). A change in commuting patterns during the COVID-19 pandemic is a likely explanation to the decreased number of DUI cases. The total number of cases where the analysis of THC was requested by the police increased by 48% from 2014 to 2019 but had decreased by 30% in 2020. The proportion of positive samples compared to the total number of cases was very stable. Taking these fluctuations into account, an upwards trend in positive DUI cases was observed from 2015 to 2019. Nevertheless, the overall results support data obtained by WBE which indicate stable cannabis use since the beginning of 2017.

Data on seized amounts of cannabis from 2014 to 2019 is presented in Fig. 7. No significant trends were observed in seized amounts of cannabis plants or marijuana, with substantial fluctuations between 2014 and 2019. Nevertheless, a 17-fold increase in seized amounts of hash was observed during the same six-year period. Although the increase in seized amounts of hash is extensive, they remain low compared with cannabis products obtained from local cultivation. A shift from illegal import of cannabis products into the country to local cultivation was observed after the financial crash in Iceland in 2008. This was determined by the low proportion of seized amounts of cannabis at the national border compared to seized amounts of cannabis from local production sites. Following the financial crash, seized amounts of hash began to decrease and have remained low since then. At the same time, an increase was observed in seized amounts of locally produced plants. With large amounts of locally cultivated cannabis the supply and demand is both stable and high in Iceland which corresponds with data by WBE showing stable use since 2017 (National Police Commissioner of Iceland, 2019).

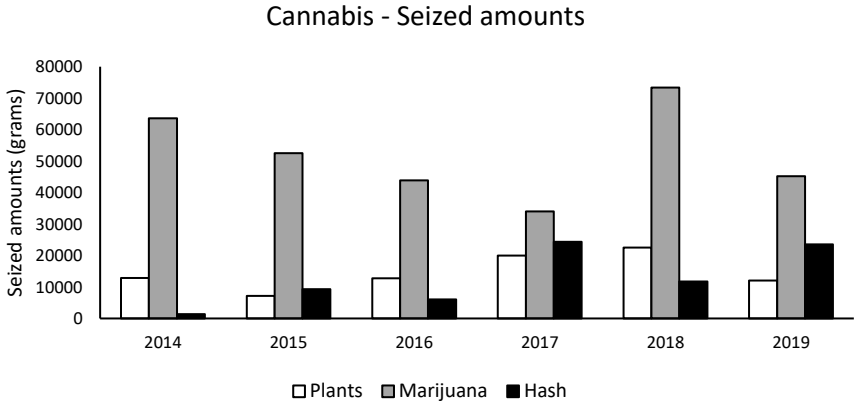

Fig. 7. Seized amounts of cannabis by the Icelandic police from 2014 to 2019.

4. Conclusions

A comparison between three indicators of illicit drug use in Iceland has been successfully achieved. By comparing multiple indicators of drug use, a more comprehensive picture of the drug problem in Iceland can be obtained.

Results show an increase in amphetamine use according to all indicators, with an increase in local production and a stable supply of the drug. Similar results were shown for methamphetamine, a relatively new drug on the Icelandic drug market, with increasing use according to all indicators, but remains low compared to amphetamine. Minimal trends were observed for MDMA with relatively stable use, but the indication of recreational use was evident. An extensive rise in cocaine use has been observed according to all indicators, likely due to increased availability of the drug and decreased prices. A decrease in cocaine use was detected during the COVID-19 pandemic indicating a change in patterns of use. Evidence of recreational use of cocaine was also apparent.

For the first time, WBE has been applied to estimate temporal trends in cannabis use in Iceland which showed comparable trends with the number of DUI cases and seized amounts. Overall, stable trends were observed in cannabis use, but an increase was detected during the COVID-19 pandemic. According to the results a shift in cannabis use was observed after the financial crash in 2008 with a large increase in locally cultivated cannabis but has remained stable in recent years.

These results show that by applying WBE provides crucial additional data on illicit drug use in Iceland in comparison with other indicators such as the number of DUI cases and seized amounts of drugs. All indicators combined painted a similar picture and provided important information on illicit drug use in Iceland.

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Paper IV

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Analysis of stimulant drugs in the wastewater of five Nordic capitals

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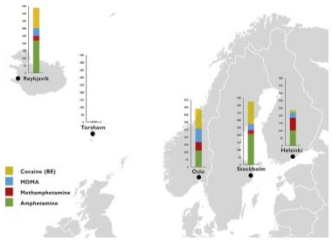
HIGHLIGHTS

- First Nordic comparison based on wastewater analysis of stimulant drugs in five capital cities
- Analytical performance was ensured by inter-laboratory comparison studies.
- Results revealed high use of amphetamines but not cocaine, compared with other European cities.
- Recreational use of cocaine and MDMA indicated by higher levels during weekends.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology is an efficient way to assess illicit drug use, complementing currently used methods retrieved from different data sources. The aim of this study is to compare stimulant drug use in five Nordic capital cities that include for the first time wastewater samples from Torshavn in the Faroe Islands. Currently there are no published reports that compare stimulant drug use in these Nordic capitals. All wastewater samples were analyzed using solid phase extraction and ultra-high performance liquid chromatography coupled to tandem mass spectrometry. The results were compared with data published by the European Monitoring Centre for Drugs and Drug Addiction based on illicit drugs in wastewater from over 50 European cities. Confirming previous reports, the results showed high amphetamine loads compared with other European countries. Very little apparent abuse of stimulant drugs was detected in Torshavn. Methamphetamine loads were the highest from Helsinki of the Nordic countries, indicating substantial fluctuations in the availability of the drug compared with previous studies. Methamphetamine loads from Oslo confirmed that the use continues to be high. Estimated cocaine use was found to be in the lower range compared with other cities in the southern and western part of Europe. Ecstasy and cocaine showed clear variations between weekdays and weekends, indicating recreational use. This study further demonstrates geographical trends in the stimulant drug market in five Nordic capitals, which enables a better comparison with other areas of the continent.
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1. Introduction

Increasing use and higher potency of drugs of abuse poses a continuing threat to human health (European Monitoring Centre for Drugs and Drug Addiction, 2016b). Illicit drug use is known to have negative effects on crime rates and can cause serious public health issues such as higher risk of premature death and transmission of infectious diseases. These effects can lead to high economic and social costs (United Nations Office on Drugs and Crime, 2016). Monitoring drug use is crucial in order to fully understand the problem and develop efficient countermeasures (European Monitoring Centre for Drugs and Drug Addiction, 2016b).

Drug use and availability is known to show both temporal and geographical variations (Kankaanpää et al., 2014; Ort et al., 2014; Thomas et al., 2012). For example, amphetamines have a higher prevalence of use in northern and eastern Europe, as opposed to cocaine in western and southern Europe (European Monitoring Centre for Drugs and Drug Addiction, 2016b). National population surveys, information on drug seizures and clinical data have traditionally been used to monitor drug consumption. According to national reports based on population surveys that have been commissioned and compiled by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), cannabis is the most commonly used illicit drug followed by amphetamines and cocaine in Denmark, Finland, Norway and Sweden (European Monitoring Centre for Drugs and Drug Addiction, 2017a, 2017c, 2017d, 2017e). Similar patterns in use have been reported for Iceland and the Faroe Islands, according to the European School Survey Project on Alcohol and Other Drugs (ESPAD) (European Monitoring Centre for Drugs and Drug Addiction, 2016a). However, these survey methods have shown significant limitations such as reporter bias, low response rates and struggle to provide information on rapidly changing trends in the drug market. Therefore, other measures are needed to compliment these methods to ensure a more comprehensive assessment (Castiglioni et al., 2014).

Wastewater-based epidemiology (WBE) is an approach where urinary excretion products are quantified to assess illicit drug use of a large population (Zuccato et al., 2005). WBE can give reliable results that may quickly reveal short and long-term trends in the scale of drug use. This approach can therefore provide a valuable source of complementary data to support more traditional epidemiological methods (Ort et al., 2014; Terzic et al., 2010; Thomas et al., 2012; van Nuijs et al., 2011; Zuccato et al., 2011). The EMCDDA has published the findings of the European inter-disciplinary network "Sewage analysis CORe group – Europe" (SCORE) (SCORE, 2017). This network brought scientists from relevant disciplines together to support research and innovation in Europe based on the analysis of biomarkers in wastewater. The main aim was to ensure that novel technologies were transferred to full-scale applications in order for authorities to be able to utilize the information gathered. Furthermore, the goal was to establish protocols for wastewater analysis by coordinating international studies with inter-laboratory comparisons. This European-wide network has organized measurements of illicit drugs in wastewater each year since 2011 in over 50 cities (Ort et al., 2014; SCORE, 2017; Thomas et al., 2012).

WBE studies have been performed in most Nordic countries to compliment other methods (Bramness et al., 2015; Kankaanpää et al., 2014; Ort et al., 2014; Östman et al., 2014; Thomas et al., 2012). Results have shown that amphetamines have been more dominant than cocaine on the stimulant drug market in Northern Europe (Bramness et al., 2015; Kankaanpää et al., 2014; Ort et al., 2014; Östman et al., 2014; SCORE, 2017; Thomas et al., 2012). Amphetamine is excreted unchanged in urine (30–74%) and is also an urinary metabolite of methamphetamine (4–7%) (Baselt, 2014). It is therefore important to assess the use of these two chemicals in parallel (Ort et al., 2014). WBE studies have demonstrated that methamphetamine use is prominent in central Europe and is reportedly produced in Lithuania or the Czech Republic before being exported to Scandinavian countries (Griffiths et al., 2008; Mackul'ak et al., 2014; Ort et al., 2014). Amphetamine use has generally been higher than methamphetamine use in the Nordic countries with the exception of Denmark where the use of both drugs is low compared with cocaine (Baz-Lomba et al., 2016; SCORE, 2017). However, in recent years Norway and Finland have also showed high methamphetamine loads with continued high loads of amphetamine in parallel (Bramness et al., 2015; Ort et al., 2014; SCORE, 2017). Trends in 3,4-methylenedioxymethamphetamine (MDMA) use, based on interview surveys, have indicated that the drug is becoming more common in Europe among young people (European Monitoring Centre for Drugs and Drug Addiction, 2016b). Most recent reports based on WBE show that the largest MDMA loads are measured in northern European countries such as the Netherlands, Belgium, Norway and Denmark (Baz-Lomba et al., 2016; SCORE, 2017). WBE studies have demonstrated that cocaine use is most prominent in western European countries such as Belgium, the United Kingdom, Switzerland, Spain and the Netherlands (Baker et al., 2014; Baz-Lomba et al., 2016; Been et al., 2016; SCORE, 2017).

For the first time, this study aims to compare and discuss trends in stimulant drug use based on WBE in the capital cities of Norway, Iceland, Finland, Sweden and the Faroe Islands. Never before have illicit drugs in wastewater been analyzed from Torshavn in the Faroe Islands. Chosen for this study were the stimulant illicit drugs amphetamine, methamphetamine, MDMA and cocaine. Due to analytical difficulties it was not possible to include cannabis in this study (Causanilles et al., 2017). By using similar sample preparation methods, instrumental analysis and data processing, it was possible to achieve harmonized results that can be compared with reports from other European countries in the SCORE network (SCORE, 2017). The analytical performance of methodologies was also evaluated by external quality control cycles, which were performed by different laboratories (van Nuijs et al., 2018).

2. Materials and methods

2.1. Chemicals, reagents and materials

The following materials were used for the preparation and analysis of samples from Oslo, Reykjavik, Stockholm and Torshavn: reference standards for eight illicit drugs and/or major metabolites were amphetamine, methamphetamine, MDMA, cocaine, benzoylecgonine (BE), and coca-ethylene. Reference standards were dissolved in methanol (MeOH) or acetonitrile (ACN) at concentrations of 1 mg/mL or 100 μg/mL. Corresponding isotope-labeled internal standards (ILIS) used were amphetamine-d_8, methamphetamine-d_{11}, MDMA-d_5, cocaine-d_3, BE-d_3 and cocaethylene-d_3 dissolved in MeOH or ACN at concentrations of 100 μg/mL. All reference standards and ILIS were purchased from Cerilliant (Round Rock, TX, USA). Standard stock solutions for reference standards were prepared at concentrations of 100 μg/mL in either MeOH or ACN. Mixed working solutions were prepared for the reference standards and the ILIS at concentrations of 1.0 μg/mL in MeOH. All standard and working solutions were stored at −20 °C. HPLC-grade MeOH was from Rathburn Chemicals Ltd. (Walkerburn, SCT, UK) and HPLC-grade ACN was from VWR Chemicals (Oslo, NOR). Ammonium hydroxide (NH_4OH) solution ≥25% in water was from Fluka - Sigma-Aldrich (Oslo, NOR) and formic acid (FA) 98–100% (p.a.) was from Merck - Millipore (Billerica, MA, USA). Oasis HLB μElution plates (30 μm) were purchased from Waters (Milford, MA, USA).

The following materials were used for the preparation and analysis of samples from Helsinki: reference standards purchased from Sigma-Aldrich (St. Louis, MO, USA) were amphetamine sulphate, cocaine hydrochloride and MDMA hydrochloride. A reference standard donated by the UN Narcotics Laboratory (Vienna, Austria) was methamphetamine hydrochloride. Reference standards and ILIS purchased from Cerilliant (Round Rock, TX, USA) were BE, amphetamine-d_6, cocaine-d_3, MDMA-d_5, methamphetamine-d_{14} and BE-d_3 dissolved in MeOH or ACN at concentrations of 1 mg/mL or 100 μg/mL. Carbon 13-labeled

internal standards purchased from Chiron AS (Trondheim, NO) were $^{13}C_6$-amphetamine sulphate, $^{13}C_6$-methamphetamine hydrochloride and $^{13}C_6$-MDMA hydrochloride. A Direct-Q® system with a LC-Pak™ from Merck - Millipore (Billerica, MA, USA) was used to purify water to a UHQ grade. A C_{18} reverse-phase silica cartridge was used to minimize interferences due to organic impurities in the mobile phase. Oasis MCX Vac RC solid phase extraction (SPE) cartridges (60 mg) were purchased from Waters (Milford, MA, USA).

2.2. Sample collection and storage

Sample collection details and map of all cities included in the study are presented in Fig. 1. Samples from Oslo were collected from the VEAS wastewater treatment plant (WTP) over seven consecutive days from March 11–17, 2016 (including a 3-day weekend composite sample and four 24 h samples). Seven scattered 24 h wastewater samples (five weekdays and two days during the weekend) were collected in Stockholm in May 2016 in the Henriksdal WTP from two separate locations, Henriksdal I and II. Sampling in Reykjavik was conducted in two WTPs, Skerjafjarðarveita and Sundaveita, which collect wastewater from the majority of the Reykjavik metropolitan area. Seven 24 h samples were collected consecutively at both locations in Reykjavik from August 17–23, 2016. Sample collection from Sersjantvíkin WTP in Torshavn consisted of seven 24 h consecutive samples from April 2–8, 2016. 24 h wastewater samples from Viikinmäki WTP in Helsinki were collected over seven consecutive days from March 16–22, 2016.

Samples from Helsinki were acidified on site after collection with hydrochloric acid (pH 2) to prevent degradation (Kankaanpää et al., 2014, 2016). Samples were transported to the National Institute for Health and Welfare (THL) in Helsinki and stored at −20 °C until analysis in April 2016. Samples from Oslo, Reykjavik, Stockholm and Torshavn were shipped frozen without acidification to the Norwegian Institute for Water Research (NIVA) in Oslo, and stored at −20 °C until analysis in September 2016 (Baz-Lomba et al., 2018).

2.3. Sample extraction procedure

Samples from Oslo, Reykjavik, Stockholm and Torshavn were pretreated according to Baz-Lomba et al. (2018) as follows: prior to extraction, influent wastewater samples were spiked with a mixed working solution of ILIS at a concentration of 500 ng/L. 1 mL of sample was centrifuged at 13000 rpm for 5 min at 4 °C and the supernatant was used for analysis. Solid phase extraction (SPE) was performed using Oasis HLB μElution plates, 30 μm. The plate was conditioned by washing and

	Name of WTP	Number of inhabitants	Month of sampling	Flow range (m^3/24h)	Sampling mode
Helsinki	Viikinmäki	800000	March 2016	283800 -353837	Volume proportional
Oslo	VEAS	607651	March 2016	250830 - 391527	Flow proportional
Reykjavik	Skerjafjarðarveita and Sundaveita	186000	August 2016	64725 - 73333	Time proportional
Stockholm	Henriksdal I and II	784000	May 2016	101500 - 136000	Flow proportional
Torshavn	Sersjantvíkin	820	April 2016	600 - 960	Time proportional

Fig. 1. Map of cities and sample collection details for the five locations included in this study.

rinsing with 1 mL of MeOH and 1 mL of purified water under suction. The supernatant of the wastewater samples after centrifugation was loaded onto the plate under suction and washed with 1 mL of ultrapure water. The plate was vacuum dried for 5 min. Analytes were eluted into a 96 well plate using 50 μL of 1% NH_4OH in MeOH, 50 μL of 1% FA in MeOH and 100 μL of MeOH. Eluents were combined and analysis was performed by injecting 37 μL into the ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) system.

Pre-treatment of samples from Helsinki was, according to Kankaanpää et al. (Kankaanpää et al., 2014, 2016), carried out as follows: 40 mL aliquots of acidified water samples were mixed with a phosphate buffer (adjusted to pH 2.5 with H_3PO_4), ILIS was added and centrifuged. Solid phase extraction was performed using Oasis MCX cartridges. The cartridges were first conditioned with 5 mL MeOH and 5 mL of purified water. The supernatant of the wastewater sample after centrifugation was loaded into the cartridges and washed with 3 mL of purified water, 2 mL of 0.01 M hydrochloric acid (pH 2) and 3 mL toluene consecutively. The cartridges were vacuum dried with a stream of nitrogen before the compounds of interest were eluted with 5 mL of MeOH: NH_3 (100:3). 140 μL of formic acid was added to acidify the solution and ionize the compounds in order to protect vulnerable basic analytes during the evaporation process. The eluate was then evaporated at 75 °C to approximately 100 μL. 50 μL of 0.2 M hydrochloric acid was added to the residue prior to injection (2 μL) into the UHPLC-MS/MS system.

2.4. Instrumental conditions

Samples from Oslo, Reykjavik, Stockholm and Torshavn were analyzed according to Baz-Lomba et al. (Baz-Lomba et al., 2018) as follows: a Waters Acquity UPLC system (Milford, MA, USA) coupled to a Waters Quattro Premier XE Micromass triple quadrupole mass spectrometer (Milford, MA, USA) was used with a T-wave collision cell. Electrospray ionization interface (ESI) was operated in positive ionization mode. Chromatographic separation was carried out using a Waters Acquity UPLC BEH C8 column, 1.7 μm, 2.1 × 100 mm (Milford, MA, USA). The column temperature was kept at 50 °C and the temperature of the sample manager was 4 °C. A constant flow rate of 0.5 mL/min was used with a mobile phase consisting of 0.1% ammonium hydroxide (solvent A) and ACN (solvent B). The UHPLC system was equilibrated with 2% of solvent B. The gradient elution started by increasing solvent B to 13% over 5 min and held for 0.5 min. Solvent B was increased to 50% over 3.5 min and to 95% in 0.5 min, held for 1.0 min. Solvent B was decreased to 2% over 0.2 min and held for 0.3 min for equilibrium of the column. The cone and desolvation gas was nitrogen, with flow rates of 50 L/h and 800 L/h, respectively. The collision gas was argon, with a flow rate of 0.15 mL/min. Other operational parameters were capillary voltage, 3.2 kV; source temperature, 100 °C and desolvation temperature, 450 °C. Limits of quantification (LOQ) for all analytes was 5 ng/L.

Instrumental conditions for the analysis of samples from Helsinki were according to Kankaanpää et al. (Kankaanpää et al., 2014, 2016) as follows: an Agilent Technologies Series 1290 Infinity LC system (Santa Clara, CA, USA) connected to an Agilent Technologies 6460 Triple Quad LC/MS tandem mass spectrometer (Santa Clara, CA, USA) was used. Jet stream ESI was operated in positive ionization mode. Chromatographic separation was performed using a Waters Acquity CSH™ C18 column, 1.7 μm, 2.1 × 75 mm (Milford, MA, USA), and a Waters Acquity CSH™ C18 VanGuard™ guard column, 1.7 μm, 2.1 × 5 mm (Milford, MA, USA), with a column temperature of 40 °C. A flow rate of 0.5 mL/min was used with a mobile phase consisting of 5 mM aqueous ammonium formate/0.05% formic acid at pH 3.4 (solvent A) and ACN (solvent B). The UHPLC system was equilibrated with 7% solvent B. The gradient started with 7% solvent B held for 1.0 min then solvent B was increased to 18.3% at 3.0 min, 32.1% at 6.96 min, 80% at 8.0 min and 95% at 10.0 min. Other operational parameters were drying gas, nitrogen (8 L/min, 305 °C); nebulizer gas, nitrogen (30 psi); sheath gas, nitrogen (12 L/min, 350 °C) and capillary voltage, 3 kV. Dynamic multiple reaction monitoring mode (dMRM) was used. LOQ's of analytes were the following: amphetamine 5 ng/L, cocaine 5 ng/L, BE 5 ng/L, methamphetamine 2 ng/L and MDMA 1 ng/L.

2.5. Calculations

The concentrations (ng/L) of the chosen compounds were analyzed in daily samples of wastewater. The concentrations were multiplied by the daily average of the wastewater flow (L/day) to achieve daily mass loads (g/day). The mass loads were normalized with respect to the population connected to the catchment area to give mg/day/1000 people for comparable results. Further details on all parameters used in the back-calculations can be found in Table S1 in the Supplementary information. Statistical analysis on the difference between two averages was performed by using the Student's *t*-test with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Amphetamine

Amphetamine was detectable in samples from all cities with the exception of Torshavn (Fig. 2). Amphetamine loads from Reykjavik (217 mg/day/1000 people) and Stockholm (208 mg/day/1000 people) were the highest, followed by Oslo (110 mg/day/1000 people) and Helsinki (101 mg/day/1000 people). Full summary of calculated amphetamine loads can be found in Table S2 in the Supplementary information.

Amphetamine is known to have dominated the stimulant drug market in Iceland in recent years according to the number of driving under the influence cases (data from the Department of Pharmacology and Toxicology, University of Iceland) and seized amounts (National Police Commissioner of Iceland, 2016). All cities except Reykjavik collected samples in the spring of 2016. Sampling in Reykjavik took place during a summer week when a festival was held and therefore does not represent a normal week. The SCORE study presented results from Reykjavik collected in March 2016, which indicate lower amphetamine loads (123 mg/day/1000 people) (SCORE, 2017), similar to those from Oslo and Helsinki. High number of social events during the summertime in Reykjavik is a likely cause for increased amphetamine use and high variability of results.

Amphetamine loads from Stockholm were high. Previously reported amphetamine loads from Stockholm in 2013 showed similar loads (215 mg/day/1000 people) indicating stable use since that time (Ort et al., 2014; SCORE, 2017). Seizure numbers indicate that amphetamine and cannabis dominate the Swedish drug market. Amphetamine seizures in the last decade seem to be decreasing in Sweden but have shown similar numbers since 2013 (European Monitoring Centre for Drugs and Drug Addiction, 2017b, 2017e). These trends support the presented findings that the use of amphetamine in Stockholm has been relatively stable in recent years.

Amphetamine loads from Oslo were in the higher range of European cities and were similar to what was reported in 2015, indicating stable availability of the drug since that time (SCORE, 2017). Amphetamine and cannabis are the most commonly seized drugs in Norway. Long-term trends indicate an increase in the number of amphetamine seizures with the highest amounts seized in 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017b, 2017d). This is in accordance with the SCORE study, which reported an increase in amphetamine loads in 2015 (SCORE, 2017).

Amphetamine loads from Helsinki were in the higher range when compared to other cities in the SCORE study (SCORE, 2017). According to Kankaanpää et al. amphetamine loads have significantly increased from 2012 to 2015 which was in accordance with driving under the influence and drug seizure data (European Monitoring Centre for Drugs and Drug Addiction, 2017c; Kankaanpää et al., 2016). The SCORE

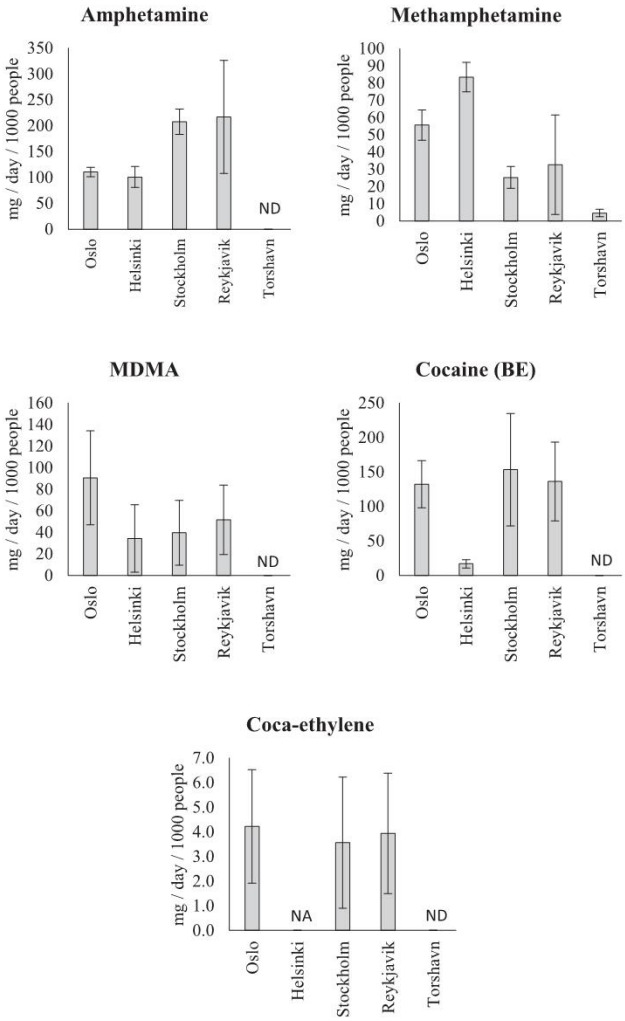

Fig. 2. Loads of illicit drugs in the different cities: Average loads standard deviation (SD) of amphetamine, methamphetamine, MDMA, cocaine (calculated form BE loads) and coca-ethylene in mg/day/1000 people. ND = not detected. NA = not analyzed.

study further indicated that amphetamine loads were relatively stable from 2015 to 2016 (SCORE, 2017).

An increase in amphetamine loads was observed during weekends in Reykjavik and Helsinki, a decrease was detected in Oslo and negligible difference in Stockholm (Fig. 3). An increase in amphetamine loads in Reykjavik by 29% during the weekend indicates that the drug is recreationally used to some extent. Nevertheless, the difference between average amphetamine loads during weekdays and weekends showed high variability, which was consistent at both WTPs. A likely cause is that sampling took place during the summertime with a high number of scattered special events, which included a festival during the weekend. A significant increase ($p < 0.05$, *t*-test) in amphetamine loads by 33% was observed in Helsinki, indicating recreational use of the drug similar to Reykjavik. Unlike other cities in this study, weekly patterns in Oslo showed a decrease during the weekend by 12%. A decrease in the population of the catchment in Oslo by approximately

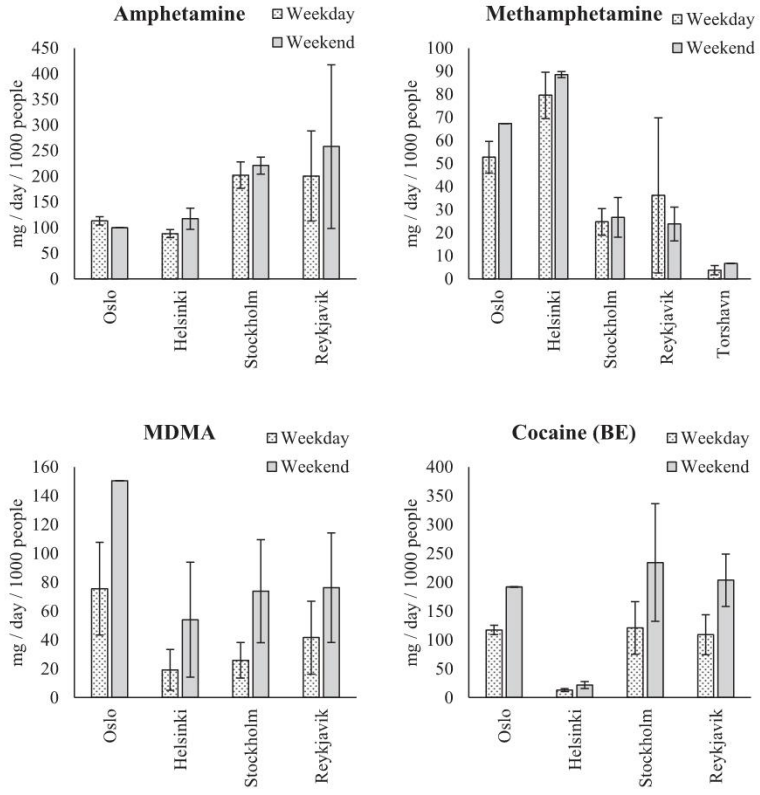

Fig. 3. Comparison of loads between weekdays and weekends: Average loads SD of amphetamine, methamphetamine MDMA and cocaine (calculated from BE loads) in mg/day/1000 people during weekends (Friday–Sunday) compared with weekdays (Monday–Thursday). Only methamphetamine was detected in samples from Torshavn.

15% has been observed during weekends, based on mobile device data (Thomas et al., 2017). This could at least partly explain the drop in amphetamine use during weekends in the area. Only one composite sample was collected during the weekend in Oslo and therefore statistical analysis on the significance of variations between weekdays and weekends was not possible. A comparison between amphetamine loads during weekdays and the weekend in Stockholm show a small increase during the weekend. This is in accordance with previous reports that have demonstrated negligible variations between days or a minimal increase during weekends in Stockholm (Baker et al., 2012; Kankaanpää et al., 2014; Thomas et al., 2012).

3.2. Methamphetamine

Methamphetamine was detected in wastewater samples from all cities included in this study (Fig. 2). Substantial geographical differences of methamphetamine loads were observed between the five sampling locations. Helsinki showed the highest average methamphetamine loads (83.4 mg/day/1000 people), followed by Oslo (55.7 mg/day/1000 people), Reykjavik (32.6 mg/day/1000 people), Stockholm (25.3 mg/day/1000 people) and Torshavn (4.6 mg/day/1000 people). Full summary of calculated methamphetamine loads can be found in Table S2 in the Supplementary information.

Methamphetamine loads from Helsinki were high in comparison with other cities in the SCORE study in 2016, below cities in Slovakia, the Czech Republic and the eastern part of Germany (SCORE, 2017). Similar loads were also measured in the Finnish cities of Espoo (89.5 mg/day/1000 people) and Jyväskylä (63.7 mg/day/1000 people) (SCORE, 2017). Kankaanpää et al. described a decrease in methamphetamine use from 2012 to 2015 in Helsinki (Kankaanpää et al., 2014, 2016). However, methamphetamine loads from Helsinki reported in the SCORE study have increased significantly ($p < 0.05$, *t*-test) since 2015, from 5 mg/day/1000 people (SCORE, 2017). These results demonstrate that there are substantial fluctuations in methamphetamine use in Helsinki, most likely depending on the availability, season, purity and price of the drug (European Monitoring Centre for Drugs and Drug Addiction, 2016b).

Methamphetamine loads from Oslo were also in the higher range when compared with other cities in the SCORE study, below Espoo, Helsinki and Jyväskylä in Finland. Bramness et al. demonstrated that the drug market in Norway has shifted from amphetamine to methamphetamine with an extensive increase since 2000, but has stabilized with a

small decrease from 2010 to 2012 (Bramness et al., 2015). This study further demonstrates a drop in the methamphetamine market in Oslo compared with previous years (Bramness et al., 2015; Ort et al., 2014; SCORE, 2017; Thomas et al., 2012). The number of methamphetamine seizures in Norway increased from 2009 to 2013 but decreased from 2014 to 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017d). These findings support the previously discussed results based on wastewater analysis.

Methamphetamine use is minimal in Reykjavik according to the number of driving under the influence cases (data from the Department of Pharmacology and Toxicology, University of Iceland) and seized amounts (National Police Commissioner of Iceland, 2016). Methamphetamine loads from Reykjavik were in the lower range, compared with other cities included in this study. Average methamphetamine loads increased by 186% from March 2016 (11.4 mg/day/1000 people) to August 2016 when compared with the SCORE study. Results from March 2016, representing a normal week, indicate that methamphetamine use in Reykjavik is low compared with other Nordic cities (SCORE, 2017). Results from Reykjavik show high variability, which is due to an unexplained spike in methamphetamine loads during the sampling week in only one of the two WTPs. Summer festivities is a likely cause for this unusual rise in methamphetamine loads on a weekday.

The average methamphetamine loads in Stockholm were low. These results are similar to previously published results on methamphetamine loads in wastewater from Sweden (Östman et al., 2014). Methamphetamine loads from Stockholm, last included in the SCORE study in 2011, were reported to be even lower (9.4 mg/day/1000 people) (SCORE, 2017; Thomas et al., 2012). Although these methamphetamine loads are both low, they show considerable shifts in the availability of the drug. Reports on the number of seizures support these fluctuations in the methamphetamine market in Sweden, although remaining low, they reached a peak amount in 2009 but have decreased since that time (European Monitoring Centre for Drugs and Drug Addiction, 2017e).

Average methamphetamine loads from Torshavn were very low. The number of people that contributed to the WTP was only 4% (820 inhabitants) of the total population of Torshavn Municipality, resulting in a high degree of uncertainty. The Sersjantvíkin WTP is the only treatment facility in Torshavn with access to influent and effluent wastewater and therefore it was not possible to collect samples that represented a larger portion of the population. It is difficult to compare these results with other countries included in this study due to these limitations. However, they indicate that the use of methamphetamine is not high in Torshavn.

Variable differences in methamphetamine loads between weekdays and weekends were observed in this study (Fig. 3). Although methamphetamine loads from Torshavn were extremely low, they show a significant increase ($p < 0.05$, *t*-test) during weekends by 81%. This gives an indication that methamphetamine is recreationally used to some degree in Torshavn. Methamphetamine loads from Oslo increased by 28% during the weekend. Only one composite sample was collected during the weekend in Oslo and therefore statistical analysis on the significance of variations between weekdays and weekends was not possible. In accordance with known patterns of use, both Helsinki and Stockholm showed limited variations in methamphetamine loads between weekdays and weekends as previous reports have described (Kankaanpää et al., 2016; SCORE, 2017; Thomas et al., 2012). Due to the high variability of results as mentioned above, it was not possible to detect significant variations between weekdays and weekends in methamphetamine loads from Reykjavik.

3.3. MDMA

MDMA was detected in samples from all cities included in this study except Torshavn (Fig. 2). Average MDMA loads were the largest in wastewater samples from Oslo (91.0 mg/day/1000 people), followed by Reykjavik (51.5 mg/day/1000 people), Stockholm (39.6 mg/day/1000 people) and Helsinki (34.2 mg/day/1000 people). Full summary of calculated MDMA loads can be found in Table S2 in the Supplementary information.

The results show that MDMA loads from Oslo have increased greatly since 2013 (from 7.4 mg/day/1000 people) (SCORE, 2017; Thomas et al., 2012). Changes in the availability and purity of MDMA in Norway have caused a recent rise in use with the highest number of seizures recorded in 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017d). This study therefore supports that MDMA use is increasing in Oslo.

The results from this study show that MDMA loads from Reykjavik were 38% higher than in March 2016 (37.4 mg/day/1000 people) when compared to the SCORE study. MDMA loads in March 2016 that represents a normal week are similar to those measured from Stockholm and Helsinki in this study (SCORE, 2017). These results indicate that MDMA is a popular recreational drug in Reykjavik with increased use during the summer time and during special event weekends.

MDMA loads from Stockholm have not been reported in the SCORE study since 2011, where it was not detected (Ort et al., 2014; SCORE, 2017; Thomas et al., 2012). The number of MDMA seizures in Sweden have increased greatly since 2009, with record-breaking numbers in 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017e). These results indicate that MDMA use in Stockholm has become more prominent in recent years.

MDMA loads from Helsinki were very similar to Stockholm. These amounts were also in the same range as MDMA loads reported from Espoo (31.9 mg/day/1000 people) and Tampere (27.1 mg/day/1000 people) in Finland, according to the SCORE study (SCORE, 2017). Kankaanpää et al. reported that MDMA use has significantly increased in Helsinki both from 2012 to 2014 and from 2014 to 2015 (Kankaanpää et al., 2016). This increase is in accordance with the SCORE study, although it also shows very similar MDMA loads in 2015 and 2016, supporting previously reported fluctuations in the availability of the drug (Kankaanpää et al., 2014; SCORE, 2017). Similar findings on the number of MDMA seizures in Finland also show an increase in recent years (European Monitoring Centre for Drugs and Drug Addiction, 2017c).

MDMA loads increased during weekends compared to weekdays with similar variability of results in all cities included in this study (Fig. 3). Weekly patterns in MDMA loads were evident in Stockholm with a significant increase ($p < 0.05$, *t*-test) by 186% during the weekend. Similar trends were observed in Helsinki with an increase ($p = 0.051$, *t*-test) by 180% during the weekend. Clear variations between days were observed in Oslo with a 99% increase in MDMA loads during the weekend. Only one composite sample was collected during the weekend in Oslo and therefore statistical analysis on the significance of variations between weekdays and weekends was not possible. MDMA loads from Reykjavik also increased significantly ($p < 0.05$, *t*-test) by 83% during the weekend. These results are in accordance with previous studies that have also shown notable variations between days in accordance with known patterns of recreational use of the drug (Kankaanpää et al., 2014; Thomas et al., 2012).

3.4. Cocaine

Cocaine is mainly excreted as the metabolite BE accounting for around 20–60% of the dose with only 1–15% excreted as unchanged cocaine (Ambre et al., 1988; Cone et al., 1998). Among the cities included in this study, both cocaine and BE were detected in samples from Oslo, Stockholm, Reykjavik and Helsinki (Fig. 2). Neither BE nor cocaine was detected in samples from Torshavn. The following discussion is based on BE loads in wastewater, representing cocaine use. Among these cities, average loads of BE were highest in Stockholm (153 mg/day/1000 people) followed by Reykjavik (136 mg/day/1000 people), Oslo (132 mg/day/1000 people) and Helsinki (16.7 mg/day/1000 people). Full

summary of calculated BE and cocaine loads can be found in Table S2 in the Supplementary information.

Stockholm had the highest average BE loads among the cities included in this study. Low BE loads from Sweden have previously been reported (48.8 mg/day/1000 people in 2011) (SCORE, 2017; Thomas et al., 2012). The number of cocaine seizures in Sweden has increased for the last ten years, reaching a peak amount in 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017e). This is in accordance with the results of this study that cocaine use is on the rise in Stockholm. However, seasonal variability and changes in the availability and purity of the drug could affect these estimates.

Average BE loads from Reykjavik in this study are comparable to what was reported in the SCORE study in March 2016 (123 mg/day/1000 people) indicating low seasonal variability of the drug (SCORE, 2017). Cocaine use in Iceland is rising according to the number of driving under the influence cases, likely in relations to the improved economic status of the country (data from the Department of Pharmacology and Toxicology, University of Iceland). Seized amounts of the drug are nevertheless low compared to amphetamine (National Police Commissioner of Iceland, 2016).

Measured average BE loads from Oslo were comparable to Reykjavik. According to the SCORE study similar BE loads from Oslo were reported in 2015 (152 mg/day/1000 people), but have decreased from a peak amount of 271 mg/day/1000 people in 2014 (SCORE, 2017). Although BE loads in wastewater from Oslo seem to be decreasing over time, the number of cocaine seizures are rising in Norway with record-breaking numbers in 2015 (European Monitoring Centre for Drugs and Drug Addiction, 2017d). This could indicate that substantial fluctuations are in the cocaine drug market in Oslo.

Helsinki had the lowest BE loads compared with other cities included in this study. The SCORE study generally showed low BE loads from Finnish cities with similar loads from Espoo (10.5 mg/day/1000 people) and Lahti (9.9 mg/day/1000 people) (SCORE, 2017). Although cocaine use is currently low in Finland, according to Kankaanpää et al. it is now rising, showing a significant increase both from 2012 to 2014 and from 2014 to 2015 (Kankaanpää et al., 2016).

Similar to MDMA, an increase in BE loads during weekends was observed in this study (Fig. 3). Changes between weekdays and weekends in BE loads from Stockholm were evident with a significant increase ($p < 0.05$, *t*-test) by 94% during the weekend. The results from Stockholm showed high variability explained by a steep increase over the weekend and a sudden decrease during weekdays. This indicates that cocaine is largely used recreationally in Stockholm. Similar trends were observed in BE loads from Reykjavik with a significant increase ($p < 0.05$, *t*-test) by 86% during the weekend compared with weekdays. Helsinki and Oslo showed comparable variations between weekends and weekdays with an increase in BE loads by 67% and 64%, respectively. The difference in BE loads from Helsinki between weekdays and weekends was statistically significant ($p < 0.05$, *t*-test). Only one composite sample was collected during the weekend in Oslo and therefore statistical analysis on the significance of variations between weekdays and weekends was not possible. These trends in BE loads support known recreational use patterns of cocaine that have also been previously reported by other countries (Thomas et al., 2012).

Large amounts of cocaine that have been dumped into the sewer system can be estimated by observing the cocaine/BE ratio. According to the maximum amount of cocaine excreted unchanged (15%) versus the minimum amount excreted as the major metabolite BE (20%), the cocaine/BE ratio should be below 0.75 if the drug is excreted through urine, and was therefore not dumped (van Nuijs et al., 2009). Partial-degradation of cocaine to BE through hydrolysis during sewer transport can also affect these measurements resulting in a lower cocaine/BE ratio (Castiglioni et al., 2013). Wastewater from Helsinki was measured to have the highest average ratio (0.65), followed by Oslo (0.51), Stockholm (0.37) and Reykjavik (0.23). These results show that estimations of cocaine in wastewater in this study are from human consumption.

The metabolite coca-ethylene is produced after the co-consumption of cocaine and ethanol (Rodríguez-Álvarez et al., 2015). Coca-ethylene accounts for 0.7% of a cocaine dose but is less active than cocaine (Baselt, 2014). Coca-ethylene was analyzed in wastewater samples from Oslo, Reykjavik, Stockholm and Torshavn. Coca-ethylene was detected in samples from all the cities mentioned above, except Torshavn, with very low loads in all instances (Fig. 2). Total average loads indicate that co-consumption of cocaine and ethanol was comparable in Oslo (4.2 mg/day/1000 people), Reykjavik (3.9 mg/day/1000 people) and Stockholm (3.6 mg/day/1000 people). Coca-ethylene was not analyzed in samples from Helsinki. Full summary of calculated coca-ethylene loads can be found in Table S2 in the Supplementary information. Comparison between BE and coca-ethylene detected in samples from these three cities showed similar geographical trends. Coca-ethylene loads in wastewater from Oslo, Reykjavik and Stockholm increased extensively during the weekend, causing high variability of results. The co-consumption of ethanol and cocaine can therefore be related to recreational use of a combination of these substances.

4. Conclusions

By using WBE, a reliable comparison between five capitals in the Nordic countries has been achieved. For the first time, information on stimulant drugs in wastewater is available from Torshavn, indicating very little stimulant drug abuse in the Faroe Islands. The results support previous findings indicating a high prevalence of amphetamines in northern Europe compared with cocaine. Clear variations between days in MDMA and BE loads support reports of the rising recreational use of ecstasy and cocaine. Temporal and geographical changes were observed that further add to current information on illicit drug use in the Nordic countries. This study enables a comparison with previously published studies in the field of WBE and illicit drug use in other European countries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.274.

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